

The Journal of Experimental Biology

EDITED BY

J. GRAY and J. A. RAMSAY

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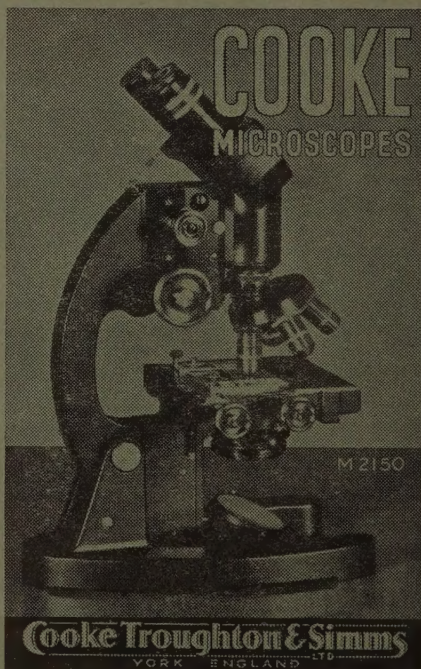
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ENZYMIC OXIDATION OF AMINES IN DECAPODS

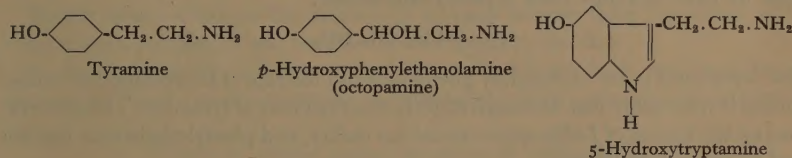
BY H. BLASCHKO AND JEAN M. HIMMS

*Department of Pharmacology, University of Oxford
and the Marine Biological Laboratory, Plymouth*

(Received 31 January 1953)

Some time ago it was shown that a high concentration of the enzyme amine oxidase was present in the liver of *Eusepia officinalis* (Blaschko, 1941); it has since been found that this is also true for *Octopus vulgaris* (Blaschko & Hawkins, 1952a). In both species the enzyme occurs in other tissues as well as in the liver.

The question arises: what is the functional significance of the enzyme in cephalopods? Tyramine, one of the chief substrates of amine oxidase, was first isolated from the posterior salivary glands of *Octopus macropus* (Henze, 1913), and it has since been shown that other substrates of amine oxidase are present in octopods. The existing knowledge has recently been reviewed by Erspamer (1952), to whom we owe much new work on this subject. He has found tyramine in the posterior salivary glands not only of *O. macropus*, but also of *O. vulgaris*; he did not find it in the gland of *Eledone moschata*. An amine closely related to tyramine was present in the glands of all three octopods; this he called octopamine and he identified it as $(-)-p$ -hydroxyphenylethanolamine. The third amine is 5-hydroxytryptamine, a substance which also occurs in mammals; it has been found in the glands of *E. moschata* and *Octopus vulgaris*. Erspamer points out that the amines occur only in extracts of the posterior salivary glands, and not in other tissue; he has never found any of these amines in *Sepia* or *Loligo*.



In *Sepia* amine oxidase had previously been found in the liver, the posterior salivary glands, the 'kidneys' and the wall of the ink sac (Blaschko, 1941). We have now made a more thorough survey of the occurrence of the enzyme in *Sepia*, and studies of amine oxidase activity in the organs of *Loligo forbesii* are also reported.

MATERIAL AND METHODS

Specimens of *Sepia* were sent from Plymouth and dissected in Oxford on the same day. The specimens were no longer alive but in reasonably good condition. *Loligo* was dissected fresh in Plymouth, and the tissues were brought to Oxford in solid CO_2 . Enzyme preparations were made by grinding the tissues in an ice-cold

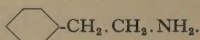
mortar and suspending them in 0.067 M-sodium phosphate buffer of pH 7.4. The extracts were used fresh, with the exception of the preparation of optic lobe from *Loligo* which served in the experiment of Fig. 1; this extract was dialysed overnight in order to reduce the 'enzyme blank'. In the experiments summarized on Table 1, semicarbazide was added in a final concentration of 0.01 M. Semicarbazide does not inhibit amine oxidase, but it prevents the further oxidation of the aldehyde formed in the enzymic reaction. The manometric determination of oxygen consumption was carried out in conical manometer flasks. Flasks of two different sizes were used, according to the amount of tissue available. The larger flasks contained in the main compartment: 1.4 ml. extract and 0.2 ml. 0.1 M-semicarbazide, in the side bulb: 0.4 ml. water or 0.05 M-substrate hydrochloride, and in the inner tube: 0.3 ml. N-KOH. In the smaller flasks the corresponding amounts were: 0.49 ml. extract, 0.07 ml. semicarbazide, 0.14 ml. water or substrate and 0.1 ml. KOH. Experiments were carried out in an atmosphere of oxygen at a temperature of either 25 or 37.5°C. Enzymic activity is expressed in terms of q_{O_2} , i.e. μ l. O_2 consumed by 100 mg. of fresh weight of tissue per hour. These figures were calculated from the initial readings, usually those during the first 15 min.

We are grateful to Dr W. Feldberg, F.R.S., for the gift of 5-hydroxytryptamine (enteramine) picrate, and to Prof. A. Th. Knoppers for that of ($\pm$)-*p*-hydroxyphenylethanolamine hydrochloride.

In the nomenclature of the organs we have followed Tompsett (1939).

EXPERIMENTS

In the earlier work on *Sepia* and *Octopus*, tyramine and *iso*amylamine had been used as the chief substrates in testing for amine oxidase activity. Both these amines have some disadvantages: *iso*amylamine is oxidized rather slowly by amine oxidase, and tyramine might also be oxidized by a phenolase. In the present experiments on *Sepia* we have therefore used β -phenylethylamine,



This compound is not oxidized by phenolases and the rate of its oxidation by amine oxidase is reasonably fast, although slightly less than that of tyramine. The observations on the tissues of *Loligo* were carried out earlier, and phenylethylamine was not used in every experiment.

Table 1 gives a list of all experiments on different tissues of *Sepia*, using 0.01 M- β -phenylethylamine hydrochloride as substrate. The incubation was carried out in the presence of 0.01 M-semicarbazide.

We have confirmed that the liver gives highly active preparations. High activity was also found in other parts of the digestive system, e.g. the pancreas, the stomach and the caecum. As in the earlier work, the extracts of the posterior salivary glands were active, but the amount of material from the anterior salivary glands did not suffice for a measurement of enzymic activity. Activity was also high in the renal appendages and in the pericardial gland. In order to obtain more material for the study of amine oxidase in the vascular system the venous and arterial hearts were

pooled; these also gave highly active extracts. Amine oxidase activity was also found in the ink sac and in the gills. Enzymic activity was also found in the gonads and various parts of the male and female genital systems.

Amine oxidase activity was found in all parts of the nervous system. The stellate, gastric, and cerebral ganglia and the optic lobes were dissected out separately, but the remaining head ganglia were pooled. These included the superior buccal, the brachial, pedal and visceral ganglia. In the eye the optic fibre layer was the only tissue with amine oxidase activity. The white body also gave active extracts.

Table 1. *Amine oxidase in organs of Eusepia officinalis*

(Substrate: 0.01 M- β -phenylethylamine hydrochloride; $t=37.5^{\circ}\text{C.}$; O_2 . Enzymic activity is expressed in terms of q_{O_2} , i.e. in $\mu\text{l. O}_2/100\text{ mg. fresh tissue/hr.}$ Semicarbazide concentration: 0.01 M.

Organ	Wt. of tissue per flask in mg.	q_{O_2}	Organ	Wt. of tissue per flask in mg.	q_{O_2}
Posterior salivary gland	245	38	Female gonads	245	19
Liver*	49	280	Nidamental gland	700	17
Liver*	140	282	Male gonads	245	26
Pancreas	245	108	Seminal vesicle	200	42
Stomach	125	103	Cerebral ganglion	245	38
Caecum	245	75	Optic lobe	245	48
Hearts	700	72	Other head ganglia	245	61
Renal appendages	245	109	Stellate ganglion	245	30
Pericardial gland	245	120	Gastric ganglion	185	35
Ink sac	125	52	Eye: lens	245	0
Skin	125	0	Eye: optic fibre layer	245	8
Muscle	125	0	Eye: outer coat	245	0
Gills	700	34	White body	245	17

* The same liver preparation was used in two experiments.

Earlier observations on the absence of enzymic activity from the muscle of the mantle were confirmed, and no activity was found in the skin.

In the experiments on *Loligo*, phenylethylamine has not been used in all experiments, and Table 2 gives data on the oxidation of the three amines used as substrates.

Table 2. *Amine oxidase in organs of Loligo forbesii*

(Substrate concentrations: 0.01 M; $t=37.5^{\circ}\text{C.}$; O_2 .)

q_{O_2} with	Tyramine	isoamylamine	β -phenylethylamine
Organ			
Anterior salivary gland	36	—	—
Posterior salivary gland	15	1	—
Liver (i)	435	—	—
(ii)	206	—	—
(iii)	77	9	30
Gills	13	0	8
Optic lobe (i)	39	7	—
(ii)	98	16	59

The liver, the anterior and posterior salivary glands, the optic lobes and the gills were examined. All these tissues gave enzymically active extracts. In addition, we have carried out one experiment at 25° C. with an extract of ink sac of *Loligo*. This experiment closely followed the arrangement used previously in the study of the ink sac enzyme in *Sepia* (Blaschko & Hawkins, 1952*a*) except that tyramine and phenylethylamine were used as substrates. The oxygen uptake during the first 10 min. with tyramine was 66 μ l. O₂/100 mg. of tissue/hr.; with phenylethylamine the corresponding figure was 22 μ l. O₂. This shows that both amines were oxidized.

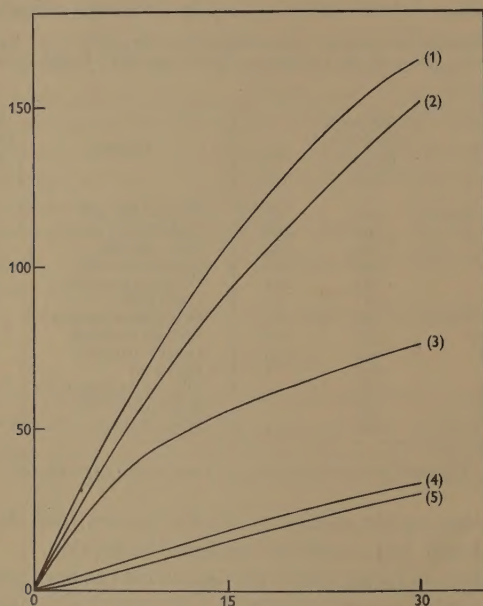


Fig. 1. Rate of oxidation of amines by a dialysed homogenate of optic lobe of *Loligo forbesii*. Initial substrate concentrations: 0.01 M. Semicarbazide concentration: 0.01 M. Gas phase: O₂; $t=37.5^{\circ}$ C. Abscissa: time in min. Ordinate: μ l. O₂ consumed. (1) tyramine; (2) tryptamine; (3) β -phenylethylamine; (4) isoamylamine; (5) ($\pm$)- p -hydroxyphenylethanolamine.

The substrate specificity of the cephalopod enzyme resembles that of the mammalian enzyme. Fig. 1 is taken from an experiment in which an extract of optic lobe of *Loligo* was incubated with various substrates. In this experiment the oxygen uptake with tryptamine was almost as fast as with tyramine, and at the end of the experiment a brown colour had developed in the flask that contained tryptamine. This is similar to observations of Pugh & Quastel (1937) on the mammalian enzyme. In another experiment, with the same extract, 5-hydroxytryptamine was oxidized almost as rapidly as tryptamine.

The ability of the cephalopod enzyme to oxidize aliphatic amines is similar to that of the mammalian enzyme. Fig. 2 shows the relative rates of oxidation in the homologous series of aliphatic monoamines $\text{CH}_3(\text{CH}_2)_n\text{NH}_2$ by a preparation of *Sepia* liver. A similar experiment was carried out with a preparation of *Loligo* liver. It can be seen that the lower members of this series were not oxidized. Oxygen uptake was observed with *n*-butylamine and the rate was maximal with *n*-heptylamine; there was little oxygen uptake with *n*-decylamine; *n*-dodecylamine and

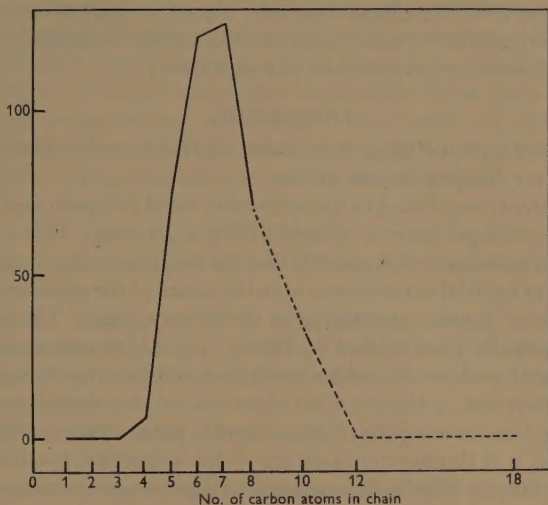


Fig. 2. Homogenate of *Sepia* liver; rate of oxidation in relation to chain length in the homologous series of aliphatic monoamines: $\text{CH}_3(\text{CH}_2)_n\text{NH}_2$. Abscissa: number of carbon atoms ($n+1$). Ordinate: $\mu\text{l.}$ of O_2 consumed by 100 mg. of tissue per hr. Gas phase: O_2 ; $t=37.5^\circ\text{C}$. Initial substrate concentrations: 0.01 M .

n-octadecylamine were not oxidized. The *Loligo* liver extract gave similar results with *n*-amylamine, *n*-hexylamine, *n*-heptylamine and *n*-octylamine, but it was inactive with *n*-butylamine and *n*-decylamine.

It is known that the mammalian enzyme does not act on histamine and the short-chain members of the series of aliphatic diamines $\text{NH}_2(\text{CH}_2)_n\text{NH}_2$, e.g. putrescine ($n=4$) and cadaverine ($n=5$), but that it does oxidize long-chain diamines (Blaschko & Duthie, 1945; Blaschko & Hawkins, 1950). The same is true for the cephalopod enzyme: *Sepia* liver did not act on histamine, putrescine and cadaverine, but tridecamethylene diamine was oxidized.

In the earlier work it was found that the enzymes of both *Sepia* and *Octopus* were inactivated when the tissues were acetone-dried: this was in contrast to the mammalian enzyme which is fully active in acetone-dried preparations. We have therefore tested the effect of acetone drying on the enzyme from *Loligo* liver and have

found that some oxidase activity is retained in acetone-dried powders. In one experiment, one *Loligo* liver, fresh weight 19.3 g., was used for the preparation of a 1 in 10 homogenate. This gave a q_{O_2} with tyramine of 170 (first 15 min.). Eight livers, fresh weight 117.75 g., were converted into 23.75 g. of acetone-dried powder. The q_{O_2} with tyramine was 165. This result indicates that about 20% of the enzymic activity was retained.

Like *Sepia* and *Octopus*, *Loligo* liver also has *D*-amino-acid oxidase activity. Since the pattern of substrate specificity of the *Sepia* and *Octopus* enzyme has already been described (Blaschko & Hawkins, 1952*b*), we have not made a thorough study of this enzyme, but we have found that the ability to oxidize *D*-methionine is retained in acetone-dried preparations of *Loligo* liver.

DISCUSSION

The experiments reported show that amine oxidase is widely distributed in the tissues of the two decapod species studied.

Very high activity was found in the alimentary canal (stomach and caecum) and its glandular appendages (salivary glands, liver and pancreas). This is analogous to what is found in mammals. It is possible that the enzyme is active in preventing the uptake of amines harmful to the animal from the lumen of the gastro-intestinal tract, but this would not explain its presence in the salivary glands. The digestive tract in *Loligo* has recently been studied by Bidder (1950); she discusses the question whether in cephalopods food particles reach liver and pancreas from the intestinal canal before absorption; with most other observers she considers this unlikely. This is probably also true for octopods (Falloise, 1906). Since amine oxidase is an intracellular enzyme, it is therefore unlikely that it has a digestive function.

As far as the salivary glands are concerned no digestive enzymes have been found in them (Romijn, 1935). The posterior salivary glands are characterized by their toxic secretion; this has been chiefly studied in octopods, but the experiments of Romijn show that the gland of *Sepia* contains a substance with a similar toxic action. It seems likely that this action of the salivary secretion is not due to tyramine and similar amines or to 5-hydroxytryptamine, and it is interesting in this connexion that these amines have never been found in the posterior salivary glands of decapods. However, the glands have amine oxidase activity and the possibility cannot be ruled out that the glands—or some other parts of the animals—contain some amines that have not yet been identified. The fact that in all cephalopods hitherto examined, octopods as well as decapods, liver and pancreas are so particularly rich in enzyme is interesting, because in these organs amines have never been found. This might be understood if we assume that in octopods the posterior salivary glands are the organs where the amines are synthesized, but that the organs particularly rich in amine oxidase are those where they exert their biological effect and where they are destroyed. In these organs one would expect to find little amine.

A special functional significance of amines is also made likely by the finding that organs not connected with the digestive tract are rich in enzyme. These are tissues

like the pericardial gland, the renal appendages, the hearts and the gills, which are connected with the vascular system.

Enzymic activity was also present in all parts of the nervous system examined. In *Loligo*, we have only examined the optic lobe, but in *Sepia* all the other large ganglia were also studied. The finding of amine oxidase in nervous tissue establishes another parallelism with the mammalian enzyme. Here again we know little about the biological significance of the enzyme.

The substrate specificity of the *Sepia* and *Loligo* enzymes is very similar to that of the mammalian enzyme. Straight-chain aliphatic monoamines were oxidized, and the optimal substrate configuration was that of *n*-amylamine and *n*-hexylamine, and the straight-chain diamine with 13 carbon atoms is also oxidized.

In conclusion, it can be said that, unless we consider amine oxidase in decapods as an interesting atavism without any biological significance, the wide distribution of the enzyme in the tissues of *Sepia* and *Loligo* suggests some function for amines in the metabolism of these species which is not yet recognized.

SUMMARY

1. The enzyme amine oxidase has been found in many organs of *Sepia officinalis* and of *Loligo forbesii*.
2. The enzyme oxidizes not only tyramine and related compounds, e.g. *p*-hydroxyphenylethanolamine ('octopamine') and β -phenylethylamine, but also aliphatic monoamines and long-chain diamines as well as tryptamine and 5-hydroxytryptamine.
3. The liver of *Loligo* contains *D*-amino-acid oxidase.

We wish to record our thanks to the Director and Staff of the Marine Biological Laboratory at Plymouth whose help has made these observations possible.

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THE MITOTIC INDEX AND INTERPHASE PROCESSES

BY P. M. B. WALKER

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The mitotic index of a cell population, defined by Champy as the *per mille* proportion of the population in any mitotic stage, has long been regarded as an important criterion of the growth and multiplication of tissues. It is commonly measured in fixed and stained specimens, and therefore represents the stage of the material at the time of fixing only. However, cells grown in tissue culture may be observed and photographed for a considerable period before any measurement is made; such observations, which have been reported elsewhere (Walker & Yates, 1952*a, b*), show that some cytochemical results may require reinterpretation.

In recent years the distribution of DNA (desoxyribonucleic acid) has been one of the principal subjects of investigation in cytochemistry, and it is clearly interesting to study the synthesis of a material so closely associated with the chromosomes in as many cell types as possible. However, although the errors in the cytochemical procedures involved have been often discussed (Caspersson, 1936; Wilkins, 1950; Glick, Engström & Malmström, 1951; Ornstein, 1952; Davies & Walker, 1953), the presentation of the results and the conclusions that may legitimately be drawn from them have not been so completely studied. Further, the conditions in which the mitotic index may be correlated with any synthesis during interphase do not appear to have been fully considered.

On the assumption that mitoses occur at random intervals, the mitotic index and the measured duration of mitosis have been used to calculate the length of interphase (Olivo & Delorenzi, 1932; Hughes, 1952). More recently, quantitative cytochemical methods applicable to single cells have been employed to study DNA synthesis during the interphase of dividing cells (Pasteels & Lison, 1950; Swift, 1950; Walker & Yates, 1952*b*); these latter methods had already supplied evidence which supported the hypothesis of DNA constancy for diploid non-dividing cells which had been deduced from the results of nucleic acid extraction from counted nuclei (Boivin, Vendrely & Vendrely, 1948; Mirsky & Ris, 1949). These cytochemical techniques are photometric, and the total amount of naturally occurring absorption or of stain is measured as the product of mean extinction and projected area. The usual method of presenting the results has been in the form of a frequency histogram, in which the amount of the substance, plotted along the abscissa, is divided into equal intervals, and the height of each section corresponds to the number of cells falling within the interval.

Two types of histogram have been figured for DNA measurements. In non-dividing cells, such as sperm heads, nucleated erythrocytes, and differentiated tissue nuclei, unimodal distributions are normally found (Swift, 1950; Ris & Mirsky,

1949; Leuchtenberger, Vendrely & Vendrely, 1951). In certain tissues containing polyploid nuclei, the values fall into unimodal classes, the mean values of which are multiples of one another. A second type of distribution has a wider range of values, which is sometimes bimodal and in which the mean of one mode is approximately twice that of the other (Fig. 1*a*). This type is found in dividing tissues and we have shown by direct observation that in the nuclei of certain tissue culture cells it is associated with the synthesis of the measured substance during interphase. There are thus intermediate values not accounted for by any overlap between the modal distributions.

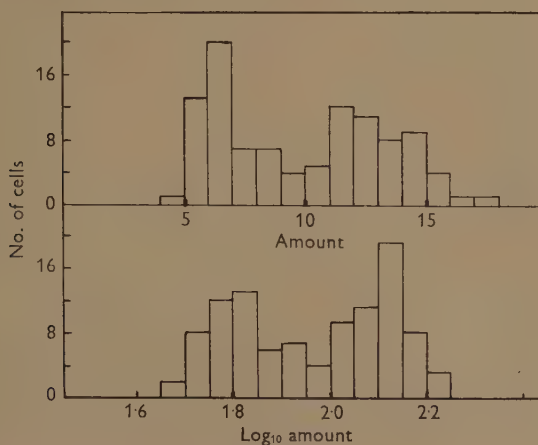


Fig. 1. Histogram summarizing the quantity of Feulgen staining material in tissue culture interphase nuclei, plotted with equal linear (*a*) and logarithmic (*b*) intervals dividing the histogram groups.

The second type of histogram is of considerable interest, since under certain conditions the relative rate of synthesis of a substance may be derived from the histogram shape. This is because the number of cells having amounts falling within a given histogram interval is a function of the fraction of interphase required for the quantity measured to increase between the interval limits. A number of factors, however, complicate the simple derivation of the synthesis curve from the shape of the histogram, of which the most important is the appearance of two daughter cells after each division, resulting in a higher proportion of younger than older cells in any growing population. A simple numerical example will make this clear. A population of 100 cells has a mitotic index of 10% and an average length of mitosis of 1 hr. For simplicity it is assumed that mitoses occur synchronously in batches at hourly intervals. Then in the first hour of observation ten cells are dividing to produce twenty daughter cells by the end of this hour. In the second hour, therefore, there will be twenty cells out of a total population of 110 within the age-group 0–1 hr. after telophase, while at the same time eleven fresh cells are dividing. During the third hour there will be twenty cells in the 1–2 hr. age-group, but the

total population will have increased to 121. Thus, as these twenty cells get older they form a progressively smaller fraction of the total population, reaching 10% at about the eighth hour.

The above example is illustrated graphically in Fig. 2. Since the mitotic index, interphase and mitotic times are interrelated, the decrease in the height of the groups will be the same for any constant mitotic index after the appropriate changes in the units have been made. A rising mitotic index will cause a more rapid decrease with increasing age, and the converse will occur with a falling index.

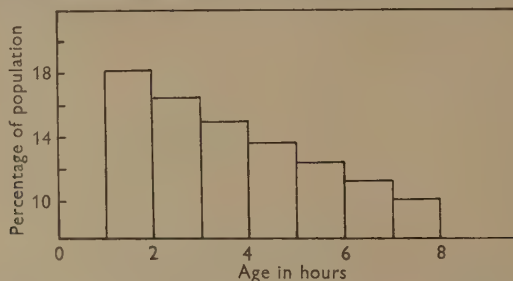


Fig. 2. Histogram showing the percentage of the cell population in different age-groups, where the mitotic index is 10% and the length of mitosis 1 hr.

It is obvious that allowance should be made for the unequal size of the different age-groups when calculating the shape of the synthesis curve. I am indebted to Prof. Longuet-Higgins, both for indicating the presence of this higher proportion of younger cells and for deriving the following expression relating the fraction of the population in each histogram group to the fraction of the generation time (interphase and mitosis) required for the measured quantity to increase between the limits of this group. It is assumed that growth in the whole population is logarithmic (i.e. that the mitotic index is constant) and that the quantity of substance synthesized after any *fraction* of the generation time (which however may itself vary) is the same for all cells.

Let us consider the histogram shown in Fig. 3, in which the ordinate represents the number of cells (n) and the abscissa the amount of substance in the cell, which ranges from m_0 to m_T , where $m_T = 2m_0$. T is the total generation time of the cell. The histogram is divided into ν groups each of equal increments μ of the substance measured. A series of difference equations may now be derived for each histogram group employing the subscript notation of the difference calculus:

$$2^{-a(m_0+\mu)/T} - 2^{-a(m_0)/T} = -\frac{1}{2}\phi(m_0 + \frac{1}{2}\mu), \quad (1)$$

$$2^{-a(m_0+2\mu)/T} - 2^{-a(m_0+\mu)/T} = -\frac{1}{2}\phi(m_0 + 1\frac{1}{2}\mu), \quad (2)$$

$$\text{to } 2^{-a(m_0+\nu\mu)/T} - 2^{-a(m_0+\overline{\nu-1}\mu)/T} = -\frac{1}{2}\phi(m_0 + \overline{\nu-1}\frac{1}{2}\mu). \quad (3)$$

ϕ is the fraction of the total population in the designated histogram group and $a(m)$ is the age of the cell when it contains the quantity m of the measured substance; thus $a(m)/T$ is the fraction of the generation time required to synthesize the quan-

tity m . $a(m_0)$ in equation (1) is zero, so $2^{-a(m_0)/T}$ becomes unity and it is therefore possible to solve this equation knowing either ϕ or a/T . This result may then be substituted in equation (2), and the process repeated until a/T becomes unity and the synthesis curve or histogram is complete.

It appears from the above equations that it is unnecessary to know the generation time and that a lengthening of this period will not affect the histogram shape provided that the initial assumptions are valid.

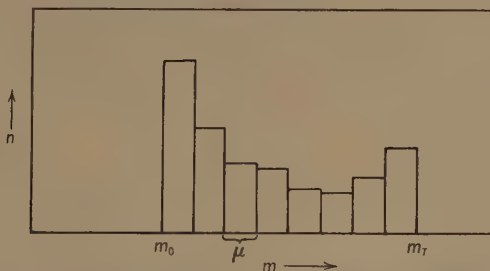


Fig. 3. An ideal histogram, where m_0 and m_T are the quantities of substance present at the beginning and end of each cell generation. μ is the extent of the interval limits and $v=5$.

These calculations are also necessarily based on the premise that the sample included in the histogram is representative not only of the population but also of all stages in synthesis. This cannot be so for cells that are not synthesizing the measured substance and whose generation time is infinite. In the rapidly dividing nuclei of chick fibroblasts grown *in vitro* we find variations both in interphase times and in rates of synthesis, and a proportion of cells that are not synthesizing DNA. Clearly this proportion of 'non-participating' cells will have to be deducted from the group m_0 (Fig. 3) before the difference equations can be applied.

Another factor, which in practice is found to influence the shape of the histogram, is the spread of values above and below the theoretical limits m_0 and m_T . This spread, which also occurs in the unimodal distributions found for non-dividing tissues, is usually ascribed to experimental error although it is also possible that individual nuclei show variation. It has the effect of artificially flattening the higher end of the histogram to a greater extent than the lower, since, while the error is probably proportional to the quantity measured, the histogram intervals are normally constant linear increments. The apparent change in shape arising from this spread of results is illustrated by the two histograms in Fig. 1, in which the same values are plotted with both linear and logarithmic increments dividing the groups.

The steps required in calculating the shape of the synthesis curve are illustrated in the following example, where it is assumed that the duration of mitosis is short compared with the generation time. Fig. 4A is a frequency histogram summarizing the results obtained from ultra-violet measurements on living tissue-culture nuclei. The divisions between the histogram groups have been chosen so that m_0 and m_T ,

whose values have been obtained from other evidence, fall on the dividing lines between the groups. It is assumed that the values outside these limits are due to experimental error,* and they have, *as an approximation*, been added to those groups within the limits as shown in Fig. 4B. The synthesis curves derived from the corrected histogram and for the same histogram with 20% non-participating cells are shown in Fig. 4C. This proportion of non-participating cells does not therefore materially affect the shape of this synthesis curve. If the increase had occurred earlier, the effect would have been more marked.

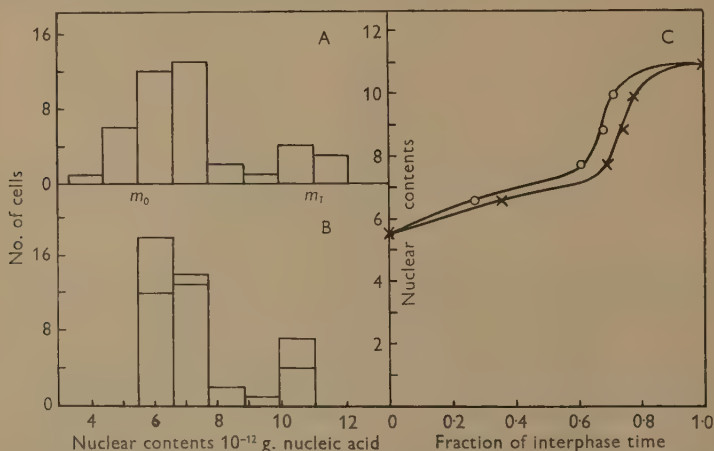


Fig. 4. A, experimentally determined histogram for the ultra-violet absorbing contents of chick tissue-culture nuclei. B, the corrected histogram. C, the synthesis curves derived from histogram B if (x) all cells are participating in the cell cycle, and (o) if 20% of the population are not so participating.

It is also possible to derive a histogram from the shape of the synthesis curve. This converse procedure may be particularly useful in determining whether the results of biochemical extraction procedures, which measure the average amount of substance in a large sample of dividing cells, are compatible with a proposed synthesis curve for that substance. Thus if we consider the synthesis curve illustrated in Fig. 5A, then the histogram in Fig. 5B will represent a random sample of the population if *all* the cells are participating in the whole cell cycle. The average nuclear content of a population of cells so distributed is approximately 6.6. This is 132% of 5.0, the latter being the average nuclear content of a cell population which is similar in all respects to the population previously considered except in that *none* of the cells is participating in the cycle. If a fraction of the population is non-participating the proportion of nuclei within the histogram group 5.5 will be

* A method for integrating the total amount of stain in nuclei has recently been completed in this laboratory. Preliminary measurements give few values outside the range m_0 to m_1 (e.g. all cells lying within the range 176 to 398 arbitrary units).

correspondingly increased and the average amount of substance in the whole population decreased. If this non-participating fraction is 20% the average nuclear content will be 6.4, which is 128% of 5.0. Hence the mean value measured in practice may be used to check the validity of a proposed synthesis curve.

It has been implicit in the preceding sections that the wide spread of results reported from photometric measurements on dividing tissue nuclei reflects an increase during interphase of the measured substance (DNA). This view has, as we have seen, been supported both by our own results obtained by direct measurement and by Swift (1950) employing photometric methods, and by other workers with different techniques (Howard & Pelc, 1950; Price & Laird, 1950). Recently, how-

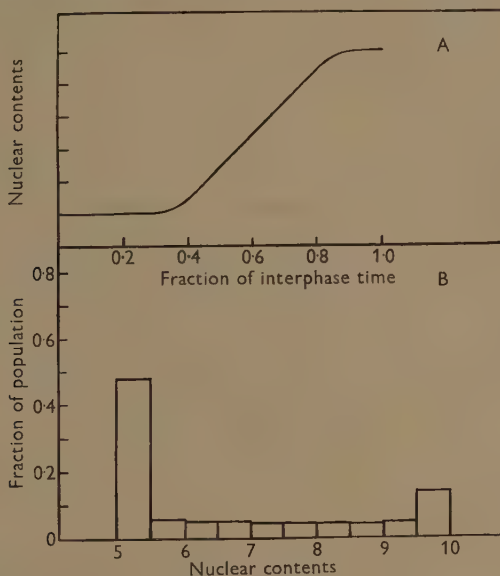


Fig. 5. A, theoretical synthesis curve for nuclear contents in arbitrary units.
B, the histogram derived from A.

ever, Moore (1952) has sought to show that this interpretation is invalid, and that the spread of values found in her material is some function of differentiation. She bases this view on her observations that in the embryonic tissues of *Rana pipiens* similar histograms are obtained from tissues with a very different mitotic index. Moore considered that if Swift's data are to be interpreted as demonstrating interphase synthesis of DNA, then there should be a positive correlation between the mitotic index and the range of values found in a frequency histogram during interphase. This is not so. If DNA doubles during interphase in dividing cells, any random sample will (experimental errors being neglected) fall within a fixed range $m_0 - m_T$, where m_0 is the amount of DNA in the post-telophase nucleus. The distribution of cells above m_0 depends only on the relative rate of DNA synthesis

(equations (1)–(3)) and is, as we have shown, independent of the total generation time. The mitotic index, on the other hand, depends on two factors; first the proportion of the cell population which participates in the whole cycle of interphase leading to division, and secondly, the relative lengths of interphase and recognizable mitotic stages. If the participating fraction does not vary, the mitotic index will remain constant provided that the ratio of interphase to mitotic time does not alter. On the other hand, if interphase time doubles while the mitotic time remains the same, the mitotic index will be halved without changing the shape of the histogram.

The essence of the confusion lies in our inability to distinguish (in material other than tissue cultures) between the two immediate causes of variation in the mitotic index. So long as interphase was treated as a resting stage and no difference could be detected between interphase cells, it did not matter whether a declining mitotic index was the result of a lengthening of interphase without a corresponding increase in the mitotic period, or whether a proportion of the cells ceased to divide while others continued to do so at the original rate. However, this distinction must be made if interphase processes are to be investigated, since a mitotic index change caused by variations in interphase and mitotic times alone will not alter the shape of the measured histogram, while a change due to an increasing proportion of non-participating cells will result in a histogram with a larger fraction of cells at m_0 .

In this short discussion we have considered certain theoretical aspects of mitotic indices and the indirect derivation of rates and times of synthesis during interphase. Clearly it would be most satisfactory if the synthesis of a substance could be followed by consecutive measurements on the same cell. This would be a laborious and slow process even if the possibility of radiation damage in ultra-violet microspectrography (Davies, 1950) did not at present make this an unreliable procedure. Photographing a culture over a period prior to making the measurements is one direct method of obtaining most of the necessary information, although variation in interphase time among the cells measured can make interpretation difficult.

In any event, it would appear that further information about the behaviour of tissue cells is necessary before synthesis curves may be accurately deduced or the weight of Moore's criticism assessed. Unfortunately, direct observation is necessarily confined to the study of cells grown *in vitro*, and the conditions found in tissue culture may not hold for tissues *in vivo*. The methods now being developed by Howard & Pelc (personal communication) employing the autoradiographic technique, may make it possible to infer the length of interphase and the proportion of participating cells in other materials. It should then be practicable to investigate how far the factors that we have discussed above apply to materials other than tissue cultures. Deductions as to the times and rates of synthesis and their correlation with the mitotic index will then rest on a surer foundation.

SUMMARY

1. Quantitative measurements of the amounts of material in a large number of individual cells are often presented as histograms, the forms of which are discussed.
2. It is shown that in a dividing tissue the rate of increase of the measured

substance in a single cell may be derived from the histogram, provided certain assumptions hold. Appropriate formulae are presented.

3. The significance of the mitotic index in relation to interphase processes is also considered. It is concluded that the relation between the shape of a histogram and the mitotic index of the tissue cannot be defined unless the durations of interphase and mitosis and the proportion of cells participating in the interphase-mitotic cycle are known.

4. The difficulty of obtaining the latter information from any material other than tissue cultures is discussed.

As mentioned in the text, my thanks are particularly due to Prof. H. C. Longuet-Higgins. I would also like to thank Dr H. B. Fell, F.R.S., Miss H. B. Yates and Dr H. G. Davies for their critical and helpful comments on the manuscript.

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STUDIES ON THE NATURE OF IMMUNITY TO HOMOLOGOUS GRAFTED SKIN, WITH SPECIAL REFERENCE TO THE USE OF PURE EPIDERMAL GRAFTS

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(With Plates 1 and 2)

INTRODUCTION

It is now generally accepted that skin does not long survive orthotopic transplantation between animals of ordinary genetic diversity; skin homografts soon break down and slough off as a consequence of an actively acquired immunity reaction on the part of the host (Medawar, 1944, 1945; Dempster, 1951). So far, all attempts to demonstrate antibodies to skin homografts by serological methods have been unsuccessful, and likewise the careful *in vitro* tests designed to reveal the existence of a specific cytotoxic activity in serum or tissue fluids against explants of the donor's skin (see Medawar, 1948*a*; Allgöwer, Blocker & Engley, 1952) have been negative. Recently, techniques have been devised for the preparation of viable pure epidermal 'grafts' comprising the superficial epidermis completely free from all dermal elements and, more particularly, of epidermal cell suspensions (Billingham & Medawar, 1951; Billingham & Reynolds, 1952). The present study represents an application of these methods to determine, firstly, whether homografts of pure epidermis can elicit and succumb to a transplantation immunity or homograft reaction like ordinary skin homografts (Part I), and secondly, as this was found to be the case, to make use of pure epidermal grafts, especially suspensions of isolated epidermal cells, in *in vitro* tests carried out in an attempt to demonstrate a specific cytotoxic activity towards a donor's cells in the blood, serum, or other derivatives from animals which had previously reacted against homologous skin from that donor (Part II). It was hoped that by using these pure epidermal preparations the tests would be made more sensitive since the generative cells of the basal or Malpighian layer would be exposed directly to the medium under test. In previous studies of this type, where thin shavings of skin have been used, a layer of mesenchymal tissue has been unavoidably interposed between the cells whose viability was being challenged and the medium whose specific activity was under examination. A shortcoming common to all *in vitro* tests so far described has been that the cells under test were in no sense isolated; thus many cells were not completely exposed to the medium.

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MATERIAL AND METHODS

Adult male rabbits and guinea-pigs from dealers' mixed stocks were used throughout. In all experiments involving rabbits the paired donors and recipients were deliberately selected from different breeds whenever possible, in the hope of achieving the greatest possible genetic diversity.

Preparation of pure epidermal grafts. Viable sheets of pure epidermis and epidermal cell suspensions were prepared by a tryptic digestion process and handled by techniques fully described elsewhere (Billingham & Medawar, 1951; Billingham & Reynolds, 1952).

Immunization of recipients. Each test made use of an independent pair of animals, a donor (*D*) and a recipient (*R*). The initial immunizing operation as used for rabbits was essentially that described by Medawar (1944, 1945, 1948*a*) except that in the present study ear skin has been used in preference to the somewhat thicker skin of the flank. Nine pinch grafts—thin disks of skin approximately 9 mm. in diameter and comprising the epidermis and the full thickness of the corium—were cut from the shaved skin of *D*'s ear and transplanted to separate recipient areas of appropriate size cut in the shaved skin of the right side of *R*'s chest. When the outward appearance of these homografts was such that breakdown was undoubtedly complete and of long standing—but in no case sooner than the 15th day after grafting—this immunization operation was repeated in the hope that the immunity elicited by the first set of homografts would be augmented; a further set of nine ear-skin homografts from *D* being transplanted to the unoperated left side of *R*'s chest. Not until at least 12 days had elapsed since it had received the second set of immunizing grafts was an animal used for experiment. Breakdown of these grafts was by then of long standing. Since there is as yet no information as to the duration of immunity against normal tissue homografts, care was taken not to allow more than 20 days to elapse after transplantation of the final immunizing grafts before carrying out a test.

The immunizing procedure for the guinea-pig was essentially as described for the rabbit, except that the grafts were cut from the shaved skin of the donor's flank. In the majority of cases two successive sets of homografts were transplanted as for the rabbit.

Details of the various operative procedures have been fully described elsewhere (Billingham & Medawar, 1951) and will be presented here only in outline.

PART I. STUDIES ON THE FATE OF PURE EPIDERMAL HOMOGRAFTS IN RABBITS

(a) *The fate of grafts transplanted to non-immunized recipients*

The experiments to be described in this section comprise seven independent tests, each making use of a paired donor and recipient. The superficial epidermis was isolated as an intact sheet by the trypsin method from each of four to six rectangular shavings of skin, about 1.25 × 0.75 cm. in lengths of side, cut from the dorsum of *D*'s ear. These 'pure epidermal homografts' were then spaced out over a rectangular full-thickness recipient area—about 6 × 7 cm. in lengths of side—prepared on the

side of *R*'s chest by stripping the skin down to the level of the vascular fascial plane that overlies the panniculus carnosus muscle. These grafts were thus transplanted in 'open style'; that is, in such a manner that each could enlarge its epithelial surface by outgrowth over the raw area separating it from its neighbour, or from the margin of the operation field.

The grafts were first examined on the 9th day post-operatively, and thereafter at 2-day intervals when biopsy specimens, which included the epithelium and some of the bed underlying it, were taken as necessary. After fixation in Formol-mercuric chloride paraffin sections were cut at 10μ and stained with Ehrlich's haematoxylin and eosin.

Results

At the first inspection, on the 9th day post-operatively, the epidermal homografts were firmly united to the underlying graft bed in all animals, and had proliferated to such an extent that a thin dry cuticular sheet could be peeled away from the surface to reveal the pinkish white, waxy surface of healthy, viable epidermis. As a consequence of the migratory outgrowth of epithelium over the developing granulation tissue, the grafts had extended considerably in area (Table 1, column 3), and had frequently lost their identity as a result of the confluence of outgrowing epithelium from adjacent grafts to produce a single epithelial sheet (Pl. 1, fig. 1). Only in one animal at this stage (Sr-119) did the appearance of the pure epidermal homografts differ significantly from that of pure epidermal autografts which Billingham & Reynolds (1952) have described in detail. In this animal the histological appearance of the biopsy specimens showed that breakdown of the grafts was far advanced.

Subsequent inspections established that this apparently healthy hyperplastic epithelium did not survive for very long. Soon its delicate pink colour deepened, becoming reddish then purple, indicating inflammatory changes in the graft bed beneath, and culminating in vascular congestion. The texture of the epithelium became increasingly delicate as it failed to make good the layers of dry cuticle which could be easily peeled off from its surface, and its attachment to the graft bed progressively weakened so that it tended to come away at inspections with the immediate graft dressings. Finally, only the smooth moist surface of the granulation tissue, which had formed the graft bed, remained on an area which a day or so previously had been surfaced by an extensive and apparently robust sheet of homologous epithelium. This sequence of changes in the outward appearance of the epidermal homografts was very constant, the only variable being the time of onset of the sequence of changes leading to the loss of the epithelium which was of the order of a few days.

Histological examination of biopsy specimens removed at 2-day intervals, commencing on the 9th day, indicated that after primary healing the grafted epithelium went through a phase of violent hyperplasia, in the course of which its thickness increased many-fold and a multi-layered cuticle was formed. Though transplanted initially to the vascular fascial layer that overlies the panniculus carnosus muscle, the grafted epidermis became separated from it by the progressive building up of an interposed layer of granulation tissue—highly vascular tissue in which young

collagen fibres differentiated (Pl. 1, fig. 2). By the 9th day, in some animals, the immediate graft bed had already been infiltrated by leucocytes, mainly lymphocytes. This infiltration was rapidly followed by dilatation of the numerous small vessels and capillaries, and distension of the young collagenous fibres by oedema fluid. There were widespread superficial haemorrhages and eventually breakdown of the vessel walls and of the native leucocytes. In other animals the sequence of events was delayed in onset. Cellular erosion took place along the interface where the Malpighian cells of the grafts abutted on to the graft bed so that this interface became obscured and lost its former histological distinctiveness. The epidermal cells became vacuolated in appearance and lost the characteristic basiphilia of their cytoplasm which became eosinophilic. Finally, as a consequence of a process of blistering, the entire necrotic remains of the epithelium became separated from the graft bed. This sequence of changes is illustrated in Pl. 1, figs. 3-5.

Though completion of the breakdown process of these grafts was conspicuous to the naked eye, the accurate timing of their survival was based on the microscopical evidence provided by the biopsy specimens. A 2-day serial sampling interval was adopted, and the chosen measure of 'survival time' (see Table 1,

Table 1. *The survival of pure epidermal homografts transplanted to raw areas prepared in the skin of the side of rabbits' chests*

Donor	Recipient	Maximum area of homologous epithelium (cm. ²)	Homograft survival time (days)
Sr-104	Sr-106	6.0	13
Sr-107	Sr-109	5.0	15
Sr-116	Sr-117	6.0	14
Sr-118	Sr-119	4.0	10
Sr-120	Sr-121	5.5	14
Sr-122	Sr-123	8.0	12
Sr-189	Sr-201	14.0	13

Median survival time 13.4 ± 1.4 days.

column 4) was taken as the time at which breakdown of the homologous epithelium was just complete (Medawar, 1944). The results, summarized in Table 1, show that the transplanted epithelium survived for at least 10 days in all animals, but in none of them did it survive beyond the 15th day. The median survival time of the pure epidermal homografts as computed from the data in Table 1 is 13.4 ± 1.4 days.

For full thickness skin homografts exchanged between rabbits of a randomly chosen heterogeneous stock in a very much higher dosage (both on the basis of the aggregate area of their superficial epithelium, and of their weight) Medawar (1944) gave a median survival time of 10.4 ± 1.1 days. This figure compares not unfavourably with the results of the present investigation, and suggests very strongly therefore that the epidermal component of a skin homograft plays a more important role than the dermis in eliciting immunity.

(b) *The fate of grafts transplanted to immune recipients*

Full thickness skin homografts, even when transplanted to specifically immunized recipients, do not undergo immediate destruction, although their breakdown is greatly accelerated. As Medawar (1944) has shown, the median survival time of such grafts in rabbits is about 6 days. It has been suggested that this delay in the fulfilment of the immune state is due to the time taken for blood vessels in the graft bed, conveying the presumptive cytotoxic agent or antibody, to penetrate the dermal portion of the graft and so gain access to its epithelial component. If this is so, then one might reasonably anticipate that pure epidermal homografts transplanted to immune animals would be acted against almost at once, since their Malpighian cells are immediately exposed to copious fluid exudates containing the presumptive antibody or cytotoxin. Moreover, a very rich capillary bed rapidly develops beneath these grafts.

Our intention was to transplant a series of small rectangular sheets of epidermis, 4–5 mm. in length of side, to a standard rectangular full-thickness bed prepared in the skin of the side of an immunized recipient's chest, and then to remove a pair of these grafts each day, commencing on the 2nd day after transplantation. One of these was for histological examination, and the other was to be grafted back to the original donor as a biological test of its continued viability. Unfortunately, preliminary trials indicated that pure epidermal homografts prepared from the skin of the donor's ear, lacking a robust cuticle, were far too delicate for this purpose, which necessarily involved repeated inspections commencing soon after operation. This difficulty was overcome by first transplanting a series of pure epidermal grafts prepared from the skin of *D*'s ear to a standard operation field cut in the skin of its own chest. By about the 15th–17th day after operation these grafts had given rise to a continuous sheet of heavily keratinized epithelium covering the central area of the operation field. From this a series of suitable pure epidermal grafts was easily obtained by defining rectangles of appropriate size by shallow incisions with a no. 15 scalpel, and then simply peeling the epidermis free from its bed with fine forceps. It may be added that although initially this epithelium was firmly united to its bed, and showed characteristic rete peg-like re-entrants into it, the union soon became very weak and the interface between the two tissues quite even, thus allowing the epidermis to be stripped off completely free from mesenchymal elements (Pl. 1, fig. 6).

In the majority of the present series of tests, instead of using paired donors and recipients, pairs of recipients shared a donor in common (see Table 2).

From the second post-operative day onwards until the 6th day, the outer dressings of *R*'s chest were removed daily and the immediate graft dressing—fine meshed tulle gras—was deflected just sufficiently to enable two grafts, including the superficial layers of the bed underlying them, to be excised. One of these specimens was fixed for histology, and the other was wrapped in a piece of sterile gauze moistened with Ringer's solution and sealed off in a small empty sterile glass vial and stored in the refrigerator at 5° C., pending the biological test of survival. This test merely

entailed transplanting the series of stored grafts in known dispositions on a standard full-thickness bed prepared on the original donor's chest. Survival of a graft was established by the appearance of epithelial outgrowth from its margins.

Results

In all animals the outward appearance of the grafts at the daily inspections proved to be completely misleading. Quite frequently, even at the first inspection on the 2nd day, their cuticles appeared damp and messy, suggesting that the grafts were already necrotic. However, the histological appearance of biopsy specimens and the results of the biological test of survival established that this was not so. There was no difficulty in assessing the survival time of the transplanted epidermal grafts: the results obtained by the grafting test completely confirmed those obtained by the histological method. In the majority of animals the grafts lived longer than 4 days, though no graft survived longer than 7 days (Table 2, column 3). It may be added that since no specimens were removed after the 6th day the assessment of a survival time of 7 days in animal Sr-156 was computed from the histological appearance of the 6-day biopsy specimen.

Table 2. *The survival of pure epidermal homografts transplanted to raw areas prepared in the skin of the side of the chest of specifically immunized rabbits*

Donor	Recipient	Homograft survival time (days)
Sr-146	Sr-147	5
Sr-146	Sr-148	5
Sr-151	Sr-152	4
Sr-151	Sr-153	3
Sr-154	Sr-155	6
Sr-154	Sr-156	7
Sr-157	Sr-158	6
Sr-208	Sr-209	6

Histologically, the problem of interpreting what took place in the grafts and in the underlying host tissue was more difficult. Sections of biopsy specimens removed on the 2nd or 3rd day after grafting showed that the thick stratified cuticular layers had become grossly distended and blistered by fluid exudates, and a heavy infiltration by polymorphs had taken place. Below this necrotic mass and very feebly united to it could be seen small patches of indolent, vegetative, feebly basiphilic epidermal cells which were intimately united to the now highly vascular bed afforded by the developing granulation tissue. Though these epithelial cells were unquestionably viable, their appearance suggested a gloomy prognosis (Pl. 1, figs. 7, 8). Contrary to expectation, however, the appearance of biopsy specimens removed subsequently suggested that these indolent strands of epithelial cells were not necessarily doomed. In some cases they took on a new lease of life, their cytoplasm becoming more strongly basiphilic. By their proliferative activity they frequently regenerated anew a more or less well-differentiated epithelium beneath

the necrotic remnants of the old one (Pl. 2, figs. 9, 10). The histological evidence strongly suggested that the grafted epithelium underwent one or more cycles of indolence during which many of the cells died, becoming eosinophilic, followed by partial regeneration of the survivors when necrotic areas of the graft became undermined by the proliferating epithelium. In some grafts there was extreme variability—patches of thick, hyperplastic epithelium being juxtaposed to areas of more or less complete epithelial breakdown. It was our impression that the condition of the grafted epithelium was a direct consequence of events in the graft bed. Though in all cases the latter had become exceedingly vascular by the 2nd or 3rd days after operation, areas of it beneath the homologous epithelium frequently showed signs of vascular stagnation in the abundant fine capillaries with an indolent phase of the epithelium above it. Frequently this apparent ischaemic phase was followed by one of activity in the capillaries in the graft bed which in turn led to a proliferative phase in the grafted epithelium. Sooner or later, however, the balance between the vascular and ischaemic states of the graft bed was tipped too far in the adverse direction, and all the cells succumbed.

Whatever the precise significance of the histological changes seen in these grafts may be, the results obtained show quite clearly that the survival of pure epidermal homografts transplanted to raw areas on immune animals is comparable with the survival of homografts of more orthodox anatomical constitution transplanted to immune animals. Thus the tissue exudates present on these graft beds have no immediate cytotoxic action. Moreover, on the basis of the present results it does not seem justifiable to attribute the survival of orthodox skin homografts transplanted to specifically immunized recipients merely to the time taken for blood vessels to penetrate them from the graft bed.

The demonstration that pure epidermal homografts both elicit and succumb to an immune reaction provided the necessary justification for making use of epidermal cell suspensions in the tests for cytotoxic activity to be described below.

PART II. ATTEMPTS TO DEMONSTRATE CYTOTOXIC ACTIVITY IN SERUM AND VARIOUS TISSUE PREPARATIONS FROM IMMUNE ANIMALS

Principle of the methods employed

In the majority of tests to be described suspensions of isolated epidermal cells prepared from a donor's skin were treated *in vitro* under various conditions with serum and various tissue preparations from the immunized recipient, and then recovered and transplanted back to the animal from which they had originally been taken as a biological test of their viability. Controls were carried out concomitantly in all tests; these entailed treatment of a similar preparation of *D*'s epidermal cells with the serum or other preparation from an animal which had not received skin homografts. It may be added that in planning the various tests it was hoped that all-or-none results would be obtained, because the techniques employed could not distinguish with certainty between different percentages of surviving cells in an epithelial population.

Two separate techniques have been employed, these being applicable to the guinea-pig and to the rabbit respectively.

(a) *The seeding technique in guinea-pigs*

There is some evidence that the melanocytes (pigmentary dendritic cells) of the mammalian epidermis (Billingham & Medawar, 1953) are more sensitive to a variety of physical and other stimuli than the neighbouring and more abundant Malpighian cells (see Taylor, 1949; Billingham & Medawar, 1952). Consequently, it seemed possible that these cells might also prove more susceptible to the action of a cytotoxic agent than their neighbours. In spotted black and white guinea-pigs with pigmented ears a readily available source of melanocytes can be obtained in the form of epidermal cell suspensions prepared from the intensely black skin of the ear—the fact that melanocytes so obtained are mixed with Malpighian cells is immaterial. After subjecting these cells to various *in vitro* treatments their continued viability can be tested simply by transferring them to a half-thickness 'graft bed' prepared in *white* skin of the donor's chest by the removal of a series of skin shavings cut so thin that the bases of the hair follicles are left intact. Areas of this sort, unlike full-thickness defects, heal rapidly by the multiple upward migration of epithelial cells accompanied by non-pigmentary melanocytes from the follicle remnants. Resurfacing of such an operation field is normally complete within 7–10 days. Survival of the grafted melanocytes is revealed by the appearance on the resurfaced bed of a number of superficial leaden-blue spots, which between about the 10th–20th days deepen in colour and extend in area (Pl. 2, fig. 11). Eventually the several expanding foci of pigmentation coalesce giving rise to a more or less continuous patch of epidermal pigmentation over the initial operation field (Pl. 2, fig. 12). Quite frequently a number of fully pigmented hairs pierce the surface since the grafting technique employed gives the melanocytes direct access to the hair follicles. A full description of the functional anatomy of the melanocyte, the 'seeding' technique for grafting these cells, and an account of the phenomenon of pigment spread in guinea-pigs' skin has been given by Billingham & Medawar (1948, 1950, 1953).

From what has already been said about the seeding technique it will be apparent that when selecting the donors it is essential that they must be recessively spotted guinea-pigs with at least one ear black and the skin of at least one side of their chest white. The colour of the recipient is of no consequence.

(b) *The seeding technique in the rabbit*

In the rabbit, unlike the guinea-pig, the skin splits naturally at the level where the corium is united to the vascular fascial plane that overlies the panniculus carnosus muscle, so that extensive full thickness graft beds free from all traces of follicular epithelium can easily be prepared. These have been referred to repeatedly in the previous section. When viable epidermal cell suspensions are transplanted to such graft beds by the method described by Billingham & Reynolds (1952) they give rise to small scattered foci of epithelium which proliferate and expand in area,

eventually coalescing to produce a more or less continuous sheet. In the present experiments two such areas of approximately equal size were prepared in the skin of the right side of the donor's chest; one of these was to receive the experimentally treated epidermal cells and the other their controls—cells treated in a similar manner with material from a non-immune rabbit. It may be added that the dressings applied both in the guinea-pig and in the rabbit were such as to preclude any possibility of cells being accidentally transferred from one area to the other. To meet the possible objection that one of the paired operation fields might be more favourable to grafted cells than the other, possibly through variation in the anatomical distribution of blood vessels etc., in each consecutive test in a series the upper and lower sites were made alternately the beds for the experimentally-treated and control cells respectively. (This precaution was also adopted in the melanocyte seeding experiments in the guinea-pig.) Primary inspections were carried out on the 9th day post-operatively, and subsequent inspections at 2-day intervals.

(c) *Withdrawal of blood*

In the rabbit 15–20 ml. of blood was withdrawn from the dilated median ear artery into a glass centrifuge tube fitted with a hard glass cannula. In the guinea-pig about 10 ml. of blood was obtained by cardiac puncture under ether anaesthesia. The blood was incubated at 37° C. in a water-bath, for not longer than 15 min., to facilitate clotting, after which it was spun for serum. All samples of blood, serum, and tissue suspensions used in the experiments to be described, were freshly prepared, full aseptic precautions being observed throughout.

(d) *Method of treatment of the epidermal cell suspensions in vitro*

In this paper the word 'treatment' has been employed non-committally; although it was found that the cells survived many of the *in vitro* procedures to which they were subjected, there were no grounds for supposing that any proliferation took place *in vitro* so that the word 'culture' was clearly inadmissible.

In the experiments to be described, whether designed to demonstrate the presence of cytotoxic agents by their effect on epidermal melanocytes in the guinea-pig, or on Malpighian cells in the rabbit, the method adopted for the *in vitro* treatment was the same. The cells were treated in small stoppered glass weighing bottles. In each individual test the epidermal cell suspension from *D*, suspended in not more than 1 ml. of saline containing sodium citrate to a final concentration of not more than 0.8%, was divided equally between two of these glass vessels containing respectively a known volume of the preparation from *R* whose specific cytotoxic effect was under test, and an equal volume of a similar preparation from an unimmunized animal to provide a control. The cells were treated *in vitro* for various lengths of time, the temperature being maintained constant at either 37° C. in an incubator or at 5° C. in the refrigerator; in a few experiments room temperature, approximately 20° C., was adopted. The effect of the lower temperature of treatment was investigated in case the cytotoxic system that it was hoped to reveal was thermolabile. It may be added that a temperature of 5° C. is known not to be

harmful to the cells of skin, this being the storage temperature normally adopted for maintaining or 'banking' this tissue in a viable state for future clinical application (see Matthews, 1945). After addition of the serum or other media to the epidermal cells in the glass weighing bottles, their contents were thoroughly mixed with the aid of a pipette. It was noted that the suspended epidermal cells soon settled out on the bottom though they did not agglutinate.

In tests conducted at 37° C. the glass weighing bottles were placed on a mechanical rocker of the type illustrated by Medawar (1948*b*). Its action, though insufficient to maintain the cells in suspension, was considered to be advantageous in promoting gaseous exchange with the medium enclosed. At the lower temperatures, continuous agitation was considered to be unnecessary in view of the presumed lower level of metabolic activity of the cells. Irrespective of the particular treatment under test and the temperature at which it was being carried out, the contents of the weighing bottles were vigorously agitated manually, on at least four separate occasions during the treatment, to resuspend the cells completely.

As the period of *in vitro* treatment was in all cases very short compared with those normally involved in tissue-culture experiments, antibiotics were not added in the majority of the experiments, and infection was exceedingly rare. In one test to be described in Exp. 2 (section 2), following Medawar (1948*a*), 9 vol. of the serum used were diluted with 1 vol. of sterile streptomycin, 200 u./ml. in Ringer, to bring the final concentration of this substance to 20 u./ml.

On completion of treatment *in vitro* the cells were resuspended, transferred to a centrifuge tube and spun down. Almost all the supernatant was then discarded, just sufficient being left to enable the cells to be taken up in a pipette in the form of a thick 'soup' for transplantation.

SECTION 1. ATTEMPTS TO DEMONSTRATE CYTOTOXIC ACTIVITY DIRECTED AGAINST THE MELANOCYTES OF THE EPIDERMIS AND OTHER CELLS IN GUINEA-PIGS

Exp. 1. Treatment of melanocytes with serum

The four independent tests that comprise this experiment simply entailed the treatment *in vitro* of a standard suspension of *D*'s epidermal melanocytes in citrated saline with an excess of freshly prepared serum from *R*, followed by their recovery and transplantation to an appropriate bed cut in the white skin of *D*'s chest. As a control a similar suspension of *D*'s cells was treated in a similar manner with serum prepared from an unimmunized animal.

In each test the melanocytes were suspended in about 5.0 ml. of serum so that the latter was in very great excess. The *in vitro* treatment was maintained for a period of 25 hr., the temperature being maintained at 5° C. in three of the tests, while in the fourth it was held at 37° C.

In all four tests the result of the 'seeding' test of melanocyte survival (see Table 3) left no doubt that a high proportion of the cells had survived the treatment, and in no animal did the appearance of the area which had received the experimentally treated melanocytes differ from that to which their controls had been grafted.

Exp. 2. Treatment of melanocytes with serum plus lymphocytes

In each of the five tests that constitute this experiment *D*'s melanocytes in suspension were divided equally between two weighing bottles to which were added respectively (a) 4-5 ml. of *R*'s serum containing lymphocytes obtained by snipping up its right axillary lymph nodes in autologous serum—i.e. the nodes draining the skin of the side of the chest to which the immunizing skin homografts were transplanted; and (b) as a control—a similar volume of serum with lymphocytes prepared from a non-immunized animal.

Table 3. *The survival (+) or non-survival (o) of epidermal melanocytes treated with serum from immune animals*

Donor	Recipient	Duration of treatment (hr.)	Temperature of treatment (° C.)	Results	
				Experimentally treated cells	Controls
Bp-1	Bp-2	25	5	+	+
Bp-5	Bp-6	25	5	+	+
Bp-7	Bp-8	26	5	+	+
Bp-9	Bp-10	26	37	+	+

Table 4. *The survival (+) or non-survival (o) of epidermal melanocytes treated with serum plus lymphocytes from immune animals*

Donor	Recipient	Duration of treatment (hr.)	Temperature of treatment (° C.)	Results	
				Experimentally treated cells	Controls
Bp-11	Bp-12	20	37	+	+
Bp-13	Bp-14	6	37	+	+
Bp-15	Bp-16	22	37	+	+
Bp-17	Bp-18	28	18	+	+
Bp-19	Bp-20	28	5	+	+

The cells were then maintained at constant temperatures for periods ranging from 6 to 28 hr. On completion of this treatment *in vitro* all the cells, i.e. a mixture of both the melanocytes and the lymphocytes, were recovered and the standard transplantation test of survival carried out.

The results of these tests are summarized in Table 4. In all five tests on both the experimental and control recipient areas the number of separate foci of pigmentary activity which developed was such as to indicate a very high percentage of survival of the treated melanocytes. In none of the tests was there any significant difference between the appearance of the experimental and control sites.

The results obtained in this experiment and the previous one indicate that epidermal melanocytes will withstand prolonged treatment *in vitro* for considerable periods at a variety of temperatures.

Exp. 3. Treatment of melanocytes with serum containing spleen cells

A preliminary series of five tests was carried out, each making use of the trio of animals as before—i.e. the donor (*D*), the immune recipient (*R*), and an unimmunized animal (*C*). After the withdrawal of blood for serum, *R* and *C* were destroyed and their spleens removed and chopped up as finely as possible with scissors in about 1 ml. of autologous serum. A further 3–4 ml. of the autologous serum was added to each and thoroughly triturated with the dissociated spleen. After the very coarse particles had been allowed to settle, the supernatants, rich in isolated cells, were taken up and added to two small weighing bottles each containing an epidermal melanocyte suspension from *D* as before. These were then placed in the 37° C. incubator, and rocked mechanically for periods ranging from 17 to 22 hr. On completion of this treatment, the melanocytes, mixed with the spleen cells, were recovered in the form of a thick brei and subjected to the 'seeding' test of viability on *D*. It was observed that in all cases the contents of the weighing bottles darkened considerably in colour during their incubation, changing from pink to brown.

Table 5. *The survival (+) or non-survival (o) of epidermal melanocytes treated with serum plus a suspension of spleen cells from immune animals*

Donor	Recipient	Duration of treatment (hr.)	Temperature of treatment (° C.)	Results	
				Experimentally treated cells	Controls
Bp-21	Bp-22	22	5	+	+
Bp-23	Bp-24	22	5	+	+
Bp-25	Bp-26	27	5	+	Not done
		27	18	+	Not done
Bp-27	Bp-28	28	5	+	Not done
		28	18	+	Not done

In none of the animals in this series of tests did any trace of melanogenesis appear on either the 'control' or the experimental sites, a result in striking contrast to that obtained when the melanocytes were treated in a similar manner with lymphocyte suspensions. One possibility was that under the conditions of our tests, e.g. at the particular temperature employed, spleen cell suspensions are toxic. Accordingly, a few control experiments were carried out in which melanocytes from a donor were treated for 27 hr. at 5° C. or 18° C., with similarly prepared spleen cell suspensions prepared from unimmunized animals. After treatment at either of these temperatures a high degree of survival of the melanocytes was obtained, as evidenced by the intense blackening of the seeded areas after three weeks.

After the conditions had been determined under which melanocytes would survive treatment with spleen cell suspensions, the series of tests summarized in Table 5 were carried out. It will be noted that controls were done only in the first two. In the remainder, by deliberate omission of the controls, it was possible to carry out two separate tests, each at a different temperature. In all cases the results

obtained indicated a very high degree of melanocyte survival and there was little to choose between the appearance of the various grafted areas 3-4 weeks after the operation.

Exp. 4. Treatment of melanocytes with whole blood

In the experiments so far described epidermal melanocytes from a donor have been shown to survive treatment with serum, with or without the addition of various cellular derivatives, from a recipient specifically immunized against the donor's skin. The present experiment was designed to test the effect of *R*'s whole blood on *D*'s melanocytes. The use of melanocyte suspensions was obviously impracticable because of the difficulty of recovering these cells from the blood clots. This difficulty was surmounted by treating the melanocytes *in situ* in sheets of pigmented epidermis prepared from the pigmented ear skin of the donor. After treating the epidermal sheets with whole blood they were transplanted to half-thickness beds cut in white skin areas on their donors' chests as before. The development of patches of epidermal pigmentation on areas so grafted was ample evidence of the survival of the melanocytes.

Paired donors and their immunized recipients were used as before. Four thin skin shavings were removed from a lightly vaselined area of pigmented skin on *D*'s ear and each was then gummed out flat, raw side outermost, on a piece of glass cover-slip made adhesive by the application of a layer of paraffin/rubber stopcock grease. Thus mounted, the shavings were incubated with the buffered trypsin solution, rinsed in Ringer's solution and the dermis removed, leaving the delicate sheet of epidermis behind mechanically supported on the cover-slip. A pair of pure epidermal grafts, so supported, was then placed on the bottom of each of two deep Petri dishes of small diameter, the epidermal sheets with their exposed basal layers being uppermost. About 5 ml. of freshly withdrawn blood from *R* was then poured over the grafts in one Petri dish, and a similar volume of blood from an animal which had not received homografts was added to the other. The Petri dishes were then placed in a moist chamber to prevent desiccation and maintained at 37° C. for periods of 24 hr. or longer. On completion of this treatment, one of each pair of epidermal sheets was carefully removed from the clot to which it was intimately united and used for the transplantation test, while the remaining graft, together with the portion of the clot to which it was attached, was cut out and fixed for histological examination.

In the three independent tests in which the cells had been incubated in contact with the blood for 24 hr., evidence of melanocyte survival was obtained in the case of both the experimentally treated cells and their controls, though the degree of survival was considerably lower than anticipated. There was no significant difference between the pigmentation appearing on the operation field to which the experimentally treated cells had been transplanted and that on which their controls had been grafted. In further experiments in which treatment was prolonged for periods up to 48 hr. and beyond, the degree of survival fell off rapidly in both the experimentally treated cells and their controls.

Histological examination of sections through treated epidermal grafts with their

attached clots left no doubt as to the intimacy of the contact between the basal layer cells, including the melanocytes, and the clot. There was no evidence of any tendency on the part of leucocytes present in the clots to migrate and concentrate in the vicinity of the homologous tissue comprising the epidermal grafts.

*Exp. 5. Treatment of ciliated tracheal cells and leucocytes
with serum and tissue preparations*

The ease with which suspensions of ciliated tracheal cells may be prepared, and the fact that ciliary activity persists for a considerable period in suspensions of these cells in physiological saline solutions, suggested to us the possibility that such material might provide a sensitive indicator capable of revealing the existence of cytotoxic activity in serum and other preparations from an immune recipient, directed against a donor's ciliated cells. Cessation of ciliary activity could easily be recognized by direct microscopy. There is a wealth of evidence that immunization by homografts is not tissue specific, and there was no reason to doubt that any antibodies acting against skin epithelium would act against tracheal epithelium.

Three independent trials were carried out, using guinea-pigs. A segment of trachea, 1.5–2 cm. in length, was dissected out aseptically from *D* and opened up by means of a longitudinal incision. Light scraping of the exposed mucosal surface detached masses of ciliated cells which were collected and suspended in Ringer's solution. A drop of this suspension of *D*'s ciliated cells was placed in each of three cavity slides. To each of these was then added several drops of one or other of the following preparations from *R*: (*a*) serum, (*b*) serum containing suspended lymphocytes, prepared by snipping up the animal's draining axillary nodes in serum, (*c*) serum containing a suspension of chopped spleen cells. As controls a similar series of cavity slide preparations was set up in which *D*'s tracheal cells were treated in a similar manner with material from an unimmunized animal. By duplicating the series of experimental slides and their controls, one series of tests was carried out at 37° C. and another at room temperature. The slides were sealed off in moist chambers to prevent evaporation. At intervals up to 48 hr. they were removed from the chambers and examined microscopically to assess the degree of ciliary activity. In nearly all of the preparations there was considerable activity for periods up to 24 hr., irrespective of the origin of the medium in which the cells were bathed and the temperature. No significant difference was demonstrable between the experimental series and their controls. In both, ciliary activity declined after 24 hr., there being no trace after 48 hr.

A somewhat similar series of controlled tests has also been carried out in which a carpet of *D*'s leucocytes, prepared on a slide by the method fully described by Wright & Colebrook (1921), were treated for periods up to 1 hr. with presumptively immune serum with or without addition of lymphocytes. On completion of the treatments fine particulate material was added in the form of bacteria (*Staphylococcus albus*), collodion, or melanin granules prepared from a mouse melanoma to test the phagocytic capability of the treated cells. There was no evidence that phagocytosis was inhibited in any of the numerous preparations examined.

SECTION 2. ATTEMPTS TO DEMONSTRATE CYTOTOXIC ACTIVITY DIRECTED AGAINST THE MALPIGHIAN CELLS OF THE EPIDERMIS IN RABBITS

In the experiments so far described it has only been possible to consider the viability of the donor's melanocytes, tracheal cells and leucocytes after their treatment with serum and other preparations from the specifically immune recipient. Though Malpighian cells were concomitantly treated with the melanocytes and subsequently grafted back to the donor with them, a serious shortcoming of the technique employed to demonstrate the viability of the melanocytes was its inability to reveal the viability of Malpighian cells. Only by transplanting Malpighian cells, whether in the form of suspensions or as intact epidermal sheets, in open style to areas completely freed from all traces of native epidermis, can the viability of these cells be established, according to whether or not they give rise to centres of epithelial outgrowth over the denuded bed. In the guinea-pig certain anatomical peculiarities of its skin preclude the preparation of the requisite type of graft bed (see Billingham & Medawar, 1952). The rabbit, however, does not suffer from this shortcoming and for this reason has been utilized in the following experiments.

*Exp. 1. Treatment of pure epidermal grafts with whole blood
from an immune recipient*

Essentially the tests now to be described are similar to those described above for melanocytes Exp. 4 (Section 1). Paired donors and their immunized recipients were used as before. Pure epidermal grafts in the form of small rectangles of hyperplastic epidermis were prepared by the indirect method described in detail on p. 20. Three of these grafts were gummed out directly, raw side uppermost, on the bottom of each of a series of small deep Petri dishes. The grafts in half the number of Petri dishes were covered with 3-4 ml. of freshly drawn blood from *R*, while those in the remainder provided controls and were covered by an equal volume of freshly drawn blood from an unimmunized animal. Pairs of Petri dishes, containing experimentally treated grafts and their controls respectively, were then placed in moist chambers and maintained at 5° C., 20° C. or 37° C. for 24 hr. On completion of these treatments the clots were carefully removed and the epidermal grafts recovered and transplanted in known dispositions on an extensive full-thickness bed prepared in the skin of the side of *D*'s chest. Survival of the treated Malpighian cells was established by the appearance of annuli of epithelial outgrowth from the grafts. On each donor-recipient pair two separate tests, with their controls, each at a different temperature, were carried out simultaneously. Twelve independent tests were thereby carried out on six donor-recipient pairs, the results being summarized in Table 6. It was found that treatment of the grafts at 5° C. and 20° C. in no way impaired their survival and there was no perceptible difference between the experimentally treated grafts and their controls.

Of the four independent tests carried out at 37° C. no survival was obtained from the experimentally treated grafts in three, but in two of the latter their controls also failed to give evidence of survival.

Table 6. *The survival (+) or non-survival (o) of pure epidermal grafts treated with whole blood from immune animals*

Donor	Recipient	Duration of treatment (hr.)	Temperature of treatment (° C.)	Results	
				Experimentally treated grafts	Controls
Sr-165	Sr-166	24	37	o	o
		24	5	+	+
Sr-171	Sr-172	24	37	o	+
		24	5	+	+
Sr-173	Sr-174	24	20	+	+
		24	5	+	+
Sr-175	Sr-176	24	37	+	+
		24	20	+	+
Sr-167	Sr-168	24	37	o	o
		24	5	+	+
Sr-161	Sr-162	24	20	+	+
		24	5	+	+

Exp. 2. Treatment of Malpighian cell suspensions with sera from specifically immunized recipients

In the extensive series of tests comprising this experiment a suspension of *D*'s epidermal cells contained in about 1 ml. of citrated saline was divided equally between two small stoppered weighing bottles containing, respectively, freshly prepared serum from *R*, and a similar volume of serum from an unimmunized animal to provide a control. Although the serum in which the cells were suspended in each individual test was always in excess, compared with the minute volume occupied by the cells themselves, its volume was deliberately varied within a lower limit of 2 ml. and an upper one of 9.5 ml. in the hope of discovering whether the amount of serum used influenced the result. The remote possibility existed that the presumed cytotoxic agent was present in very low concentrations so that by having a great excess of serum there might be a better chance of revealing its existence if it had a specific affinity for the donor's cells. The tests were carried out either at 37° C. or at 5° C., a single one being carried out at 20° C. On completion of the tests the experimentally-treated cells and their controls were recovered and distributed as evenly as possible over the central areas of two extensive full-thickness beds of approximately similar dimensions—about 6 × 4 cm.—prepared on the side of *D*'s chest. Both sites were liberally dusted with sterile sulphadiazine powder before the dressings were applied.

Inspection of the grafted areas was first carried out on the 9th day post-operatively, and thereafter at 2-day intervals until the 15th day. Further inspections were not made after this time since it gave ample opportunity for all epidermal cells which had survived the *in vitro* treatment to reveal this fact by producing foci of epithelial outgrowth.

Although it was hoped that all-or-none results would be obtained, the outcome

of the experiments already described indicated that this was most unlikely. Preliminary trials conducted with untreated epidermal cell suspensions in rabbits had established that after these had been 'seeded' over an extensive full-thickness bed they gave rise to numerous small scattered foci of keratinized epithelium, easily visible to the naked eye by about the 9th day post-operatively. These, as a result of their proliferative and migratory activity, eventually coalesced to produce a large central patch of epithelium of somewhat irregular outline, which in turn became united to the ingrowing native epithelium from the margins of the bed and so lost its individuality.

Since the number of individual foci of epithelial outgrowth which appeared on the bed, and the extent of the epithelial surface which they had given rise to by the 13th day must have been roughly proportional to the number of surviving cells or clumps of cells applied initially, this was made the basis of the method of scoring adopted in Table 7. Every attempt was made to keep the amount of cell suspension used in each test constant by removing approximately the same aggregate area of skin shavings for its preparation. Once such a suspension had been prepared its exact division into two equal portions for an individual test with its control presented no difficulty.

A total of twenty-seven independent tests, each with its own control, was carried out. In two of the tests the donors died before the results could be assessed, and in four both the experimentally treated sites and their controls were found to be infected at the first inspection, and the animals were destroyed. The results of the twenty-one remaining tests with their controls, in which both areas were perfectly clean and healthy from the primary inspection onwards, are summarized in Table 7.

In the first six tests, instead of using paired donors and recipients as before, pairs of animals were used in such a way that each stood as both donor and recipient to the other. In these tests conducted at 37°C ., and in which treatment with the serum was not prolonged beyond $3\frac{1}{2}$ hr., both the treated cells and their controls gave rise to extensive areas of epithelium when transplanted back to *D*, and in no case was there any significant difference between the appearance of epithelium arising from experimentally treated cells and that from their controls. However, in a series of five tests at the same temperature, but in which treatment was maintained for not less than 23 hr., there was a very obvious difference between the experimental sites and their controls in three, and in two of these no trace of epithelium was obtained from the experimentally treated cells. The single test conducted at 20°C . also revealed a striking difference between the appearance of the two grafted areas. Of the nine tests carried out at 5°C ., involving treatment of the cells for periods ranging from 22 to 45 hr., only one failed to reveal a significant difference between the areas to which the experimentally treated cells and their controls had been grafted respectively. In four of these tests the experimentally treated cells completely failed to give rise to a trace of epithelium. It may be emphasized that in not a single test of the twenty-one summarized in Table 7 was the outcome such that the epithelium arising from the experimentally treated cells was superior to that arising from their controls; any differences that could be distinguished were always

in the opposite direction, the controls giving evidence of a higher degree of survival than the experimentally treated cells (see Pl. 2, figs. 13, 14). Examination of the data presented in Table 7 reveals that prolongation of the *in vitro* treatment beyond about 23 hr. did not result in an improvement of the result. For example, in tests nos. 15 and 19 at least some cells survived after treatment with the immune sera for periods of 30 and 45 hr. respectively, yet in tests 11 and 13 there was no evidence that cells survived after the much shorter treatments of 23 and 22 hr. respectively.

Table 7. The fate of epidermal cell suspensions after treatment with immune serum

Test	Donor	Recipient	Volume of serum (ml.)	Duration of treatment (hr.)	Temperature of treatment (° C.)	Results*	
						Experimentally treated cells	Controls
1	Sr-88	Sr-89	4.5	1	37	+++	+++
2	Sr-89	Sr-88	4.5	2	37	+++	+++
3	Sr-94	Sr-95	4.5	3	37	+++	+++
4	Sr-95	Sr-94	4.5	2	37	+++	+++
5	Sr-99	Sr-100	7.0	2½	37	+++	+++
6	Sr-100	Sr-99	5.0	3½	37	+++	+++
7	Sr-165	Sr-166	2.0	24	37	+	++
8	Sr-167	Sr-168	2.0	24	37	o	+++
9	Sr-171	Sr-172	3.5	27½	37	+++	+++
10	Sr-206	Sr-207	5.0	29	37	+++	+++
11	Sr-225	Sr-226	5.5†	23	37	o	+++
12	Sr-175	Sr-176	4.0	29	20	+	+++
13	Sr-161	Sr-162	2.0	22	5	o	+++
14	Sr-173	Sr-174	3.0	27½	5	o	++
15	Sr-178	Sr-179	6.0	30	5	++	+++
16	Sr-180	Sr-181	8.0	28	5	++	++
17	Sr-182	Sr-183	9.5	29	5	+	+++
18	Sr-184	Sr-185	4.0	29	5	o	++
19	Sr-187	Sr-188	9.5	45	5	+	+++
20	Sr-191	Sr-192	8.5	29	5	o	++
21	Sr-193	Sr-194	7.5	26	5	+	+++

* Scoring of cell survival: o = no trace of epithelial survival on operation field 15 days after 'seeding' operation; + = only a very low degree of epithelial survival—as from a single minute focus of outgrowth; ++ = intermediate degree of survival—less than that normally obtained from untreated cells; +++ = high degree of survival, such as that normally obtained from untreated cells.

† Contained streptomycin.

The influence of temperature has not been conclusively established owing to the small number of tests carried out for 23 hr. or longer at 37° C. Certainly it can safely be stated that the results obtained with treatment at 37° C. are no better than with treatment at 5° C.; if anything there is a slight indication that the lower temperature may be the better of the two. Variation in the volume of the serum was clearly without effect, small volumes of immune serum appearing to be just as effective as double or even quadruple these volumes in inhibiting wholly or partially the capacity of the experimentally treated cells to give rise to epithelium on transplantation.

One observation that may yet prove to be of some significance was that in the majority of those tests where there was a marked difference between the amount of

epithelium resulting from the experimentally treated cells and that from their controls, the first outward indication of surviving epithelium on the experimental site was nearly always delayed to some extent compared with that on the control. Frequently there was ample evidence of cellular survival on the control site at the 9-day inspection, whereas evidence of a small degree of survival from the experimentally treated cells was not obtained until either the 11th or 13th day inspections.

DISCUSSION AND CONCLUSIONS

The evidence presented in Part I of this paper shows that when homografts of pure epidermis, free from all trace of dermis, are transplanted orthotopically between unrelated rabbits they incite and succumb to an immunity reaction in the same way as skin homografts of more familiar anatomical constitution. Rogers (1950) has suggested that pure epidermal grafts which resemble cornea and cartilage in having no direct or inherent blood supply—their nutritional requirements being met by diffusion from the blood supply in adjacent tissue—might be expected to survive homotransplantation as grafts of these other tissues do; this view is clearly erroneous. It is of some interest that the distinctive and characteristic sequence of pathological changes in their dermis that accompany the process of breakdown of ordinary skin homografts, also take place in the differentiating granulation tissue of *host* origin that forms the bed of a pure epidermal homograft. This tissue becomes heavily infiltrated with round cells, mainly lymphocytes; the young differentiating collagenous fibres become separated by oedema fluid; the contents of the vessels stagnate and eventually there is breakdown of their endothelium; the leucocytes also die. The median survival time of these pure epidermal homografts is only very slightly greater than that of orthodox skin homografts of comparable surface area, a finding that lends strong support to the belief that the epidermal component plays a far greater role than the dermis in eliciting an immunity reaction. Billingham & Boswell (1953) have presented evidence that strongly suggests that the epithelial component of corneal homografts in rabbits is antigenically more effective than the stroma when such grafts are transplanted heterotopically to sites normally occupied by skin.

When pure epidermal homografts were transplanted to vascular beds prepared in recipients already immunized against their donor's skin, their survival was found to be curtailed, but not to the extent that might have been anticipated on the grounds that in such grafts the basal layer cells were directly and intimately exposed to the action of the host's serum. This suggests that tissue exudates and serum present in the graft bed are not in themselves immediately inimical to potentially susceptible homologous cells exposed to them; indeed there was frequently evidence that at least some proliferative activity took place in such grafts before their breakdown was complete.

Of the various experiments described in Part II which have been carried out in the hope of demonstrating, on the basis of a transplantation test of survival after prolonged *in vitro* treatment, the existence of a specific inimical activity in whole blood or serum from a specifically immunized recipient, with or without the

addition of cellular components from the reticulo-endothelial system, only one has been successful. The remainder have been described in detail because it is felt that the negative result is of genuine significance for the understanding of the nature of the immunity reaction. It has been found that treatment of a suspension of a donor's epidermal cells for a period of 22 hr. or longer with excess serum from a rabbit specifically immunized against that donor's skin, in the majority of tests partially or completely suppressed their capacity to produce epithelium when they were recovered and transplanted back to an appropriate bed on the animal from which they were removed. There was no evidence that this treatment was any more effective when carried out at 37° C. than at 5° C., nor did prolongation of the treatment beyond 24 hr. enhance the effect. Controls were provided by treatment of a similar quantity of *D*'s epidermal cells with normal homologous serum from an animal which had been immunized neither against *D*'s cells nor any others. Controls of this type must surely have been adequate to reveal the existence of any non-specific activity in rabbit's serum. It may be added that a second type of control, the treatment of a rabbit's epidermal cell suspension with serum derived from a recipient specifically immunized against a donor other than itself, is not acceptable, as Medawar (1948*a*) has emphasized, for although the skin immunity reaction is strongly donor-specific (see Medawar, 1946*a*) the second donor might well happen to possess at least some antigens in common with the first, but not present in the recipient. In that event some degree of immunity would be directed against its own cells.

The apparent non-susceptibility exhibited by suspensions of epidermal melanocytes in the guinea-pig when treated with immune serum under similar conditions is surprising. One possibility is that these cells, contrary to our initial assumption, are in fact more resistant than Malpighian cells. An alternative but less probable possibility is that melanocytes are susceptible but that after transplantation to an appropriate bed cut in white skin to test their survival they succeed in 'infectively' inoculating the non-pigmentary melanocytes of the white hair bulbs with some cytoplasmic ingredient, so conferring upon them the faculty of melanogenesis (see Billingham & Medawar, 1948, 1950) before their eventual destruction or inactivation.

The question as to the nature and mode of action of the factor demonstrable in a specifically immune serum by the combined *in vitro*-*in vivo* procedure described must now be discussed. It may be stated at the outset that exhaustive attempts to differentiate microscopically between cells, which had been treated *in vitro* with immune sera and their controls, were without success and the application of supravital staining was to no avail. That some subtle constitutional change had been wrought in the experimentally treated cells could only be inferred from the fact that their capacity to produce epithelium on grafting to *D* was impaired or destroyed. One possibility, the simplest and probably that which embodies a major portion of the truth, is that we have succeeded in demonstrating the existence of a protective iso-antibody of the type that Gorer (1942), using an essentially similar technique as well as immunological ones, has shown to be produced in response to transplants

of homologous leukaemic cells in mice. It may be supposed that this protective antibody is at least partially responsible for the regression of skin homografts *in vivo*. Strictly speaking, the use of the term 'cytotoxin' is at present unjustified, since the method we have used can by its nature yield no information as to whether the susceptible cells were actually killed or not.

The failure of previous workers to demonstrate the regression of skin explants from a donor rabbit when cultivated in a medium composed of serum with or without the addition of tissue extracts or mesenchymal tissues from a highly immunized recipient may be explicable on the grounds that the exposure of the donor's cells to the presumed antibody was of insufficient intimacy to affect the well-being of the majority of cells. Another possibility, suggested by Medawar (1948*a*) and certainly not excluded by the results of our own experiments, is that immune media affect skin epithelium *in vitro* only in such a way as to expedite its breakdown on subsequent grafting; i.e. that tissue breakdown requires the co-operation of a specific antibody with vascular-inflammatory changes only demonstrable *in vivo*. Woglom's (1933, 1937) studies with transplanted rat sarcomata support this view, as do Kidd's (1946) findings that a preliminary incubation of Brown-Pearce rabbit carcinoma tissue in a medium containing its specific antibody reduced or suppressed its growth on transplantation but did not effect any alteration in the appearance of the cells when incubated *in vitro* with them.

The finding that 'second set' pure epidermal homografts survive transplantation to heavily immunized recipients for as long as 4-6 days is perfectly consistent with the existence of the circulating antibody-like agent. The latter only exerted a demonstrable effect on *even* dissociated epidermal cells after they had been exposed to excess 'immune' serum for 22 hr. or longer.

A serious shortcoming of any attempt to explain the breakdown of skin homografts *in vivo* as a consequence of a simple antigen-antibody reaction is that this does not take into account the fact that regression of homografts is accompanied by the destruction of such *native* lymphocytes and other leucocytes as may have penetrated them, as well as the native cells comprising the vessels that grow into the grafts. Our own observations (see p. 19) demonstrate this important point very clearly.

Medawar (1946*b*) and Burnet & Fenner (1949) have drawn attention to certain features of tissue transplantation immunity that qualify it for admission into the general category of tissue sensitization phenomena. Burnet & Fenner have suggested that as a working hypothesis the mechanism involved in immunity to skin homografts may be similar to that postulated for tuberculin hypersensitivity. Mitchison's (1953, 1954) recent demonstration that passive transfer of immunity to a lympho-sarcoma in mice can be achieved by the transfer of lymph nodes from immune animals but not by means of their serum or peritoneal exudates is particularly relevant.

In addition to the circulating antibody-like agent demonstrated in the present study, there may be a cell-bound component, possibly the antibody itself, as in the Arthus reaction, whose contact with antigens liberated from the donor's cells in a homograft in some way brings about the local liberation of non-specific

pharmacologically active substances which are responsible for the necrosis of the graft. It has been suggested (Gorer, 1947) that other factors than antibodies may be of importance in determining the fate of transplanted tumours.

Certain types of sensitization reaction can be inhibited by the administration of anti-histamine agents (Halpern, 1950), and at least one inconclusive clinical report has appeared of an attempt to prolong the life of a skin homograft by administration of pyribenzamine (Foster & Hanrahan, 1948). These facts prompted us to investigate the effect of phenergan (promethazine hydrochloride) on the fate of skin homografts in the rabbit and in the guinea-pig. In neither of these species did the administration by intramuscular or subcutaneous injection of dosages as high as 50 mg./kg. daily, subdivided into two equally spaced injections, with or without the concomitant local application of phenergan cream (containing promethazine hydrochloride 2%, w/w) to the grafted area, have the slightest detectable effect in prolonging the expectation of survival of the homografts. Marconi (1950) reported failure to obtain significant prolongation of the survival of skin homografts in guinea-pigs by the administration of neo-antergan.

SUMMARY

1. Homografts of pure epidermis, i.e. of skin freed from all dermal elements, survive their orthotopic transplantation between unrelated rabbits for 10–15 days. They elicit and succumb to a transplantation immunity reaction just as ordinary skin homografts do.

2. The distinctive sequence of histological changes that take place in the dermis of a skin homograft during the course of its breakdown take place in the host's *own* young fibrous tissue when this forms the bed underlying pure epidermal homografts.

3. Although the breakdown of pure epidermal homografts is accelerated when they are transplanted to specifically immunized recipients, they normally survive for from 4–6 days. It is inferred that the serum and tissue exudates present in the bed to which such grafts are transplanted have no immediate inimical effect on the susceptible Malpighian cells when these are intimately exposed to them.

4. The possible application of pure epidermal grafts, and more particularly of dissociated epidermal cells, has been investigated in attempts to demonstrate a specific toxic action against a donor's cells of the blood, serum, and other derivatives of specifically immunized animals. After treatment *in vitro*, the 'vitality' of the epidermal grafts or cells was tested by transplanting them back to the animal from which they originated.

5. It was found that epidermal melanocytes of guinea-pig's skin are unaffected by prolonged treatment *in vitro* with 'immune' blood and serum, with or without the addition of lymphocytes or spleen cells.

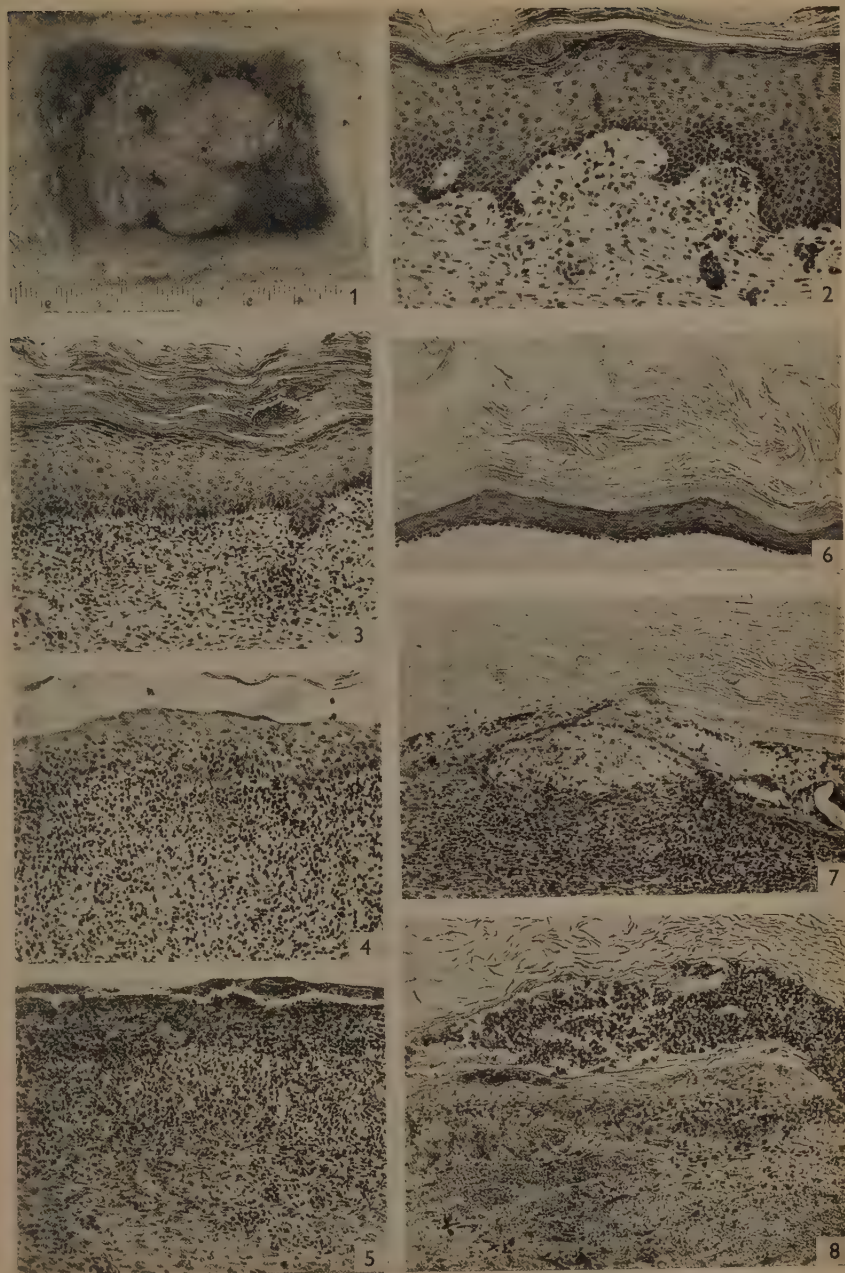
6. The capacity of dissociated Malpighian cells in the rabbit to give rise to epithelium after grafting was completely or partially inhibited by treating them *in vitro* with excess immune serum for 22 hr. or longer at either 5° C. or 37° C.

7. It is suggested that this effect indicates the presence of a protective iso-antibody in the serum of rabbits which have reacted against skin homografts. The probable role of this antibody in the breakdown of skin homografts *in vivo* is discussed.

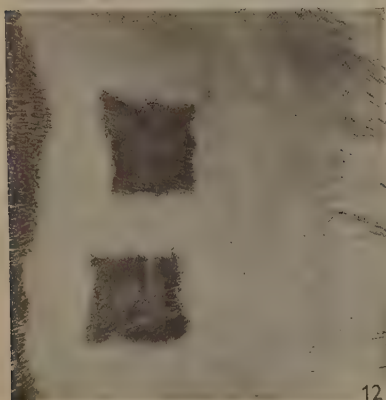
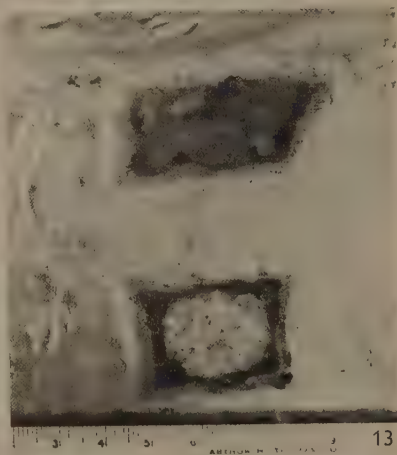
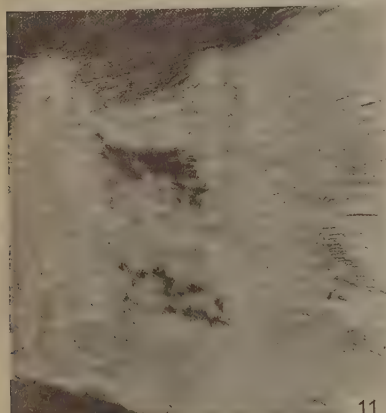
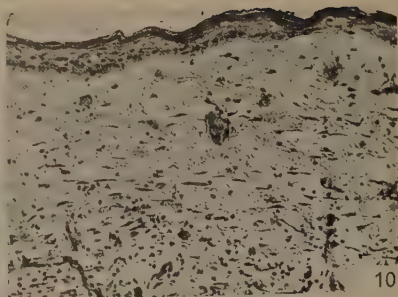
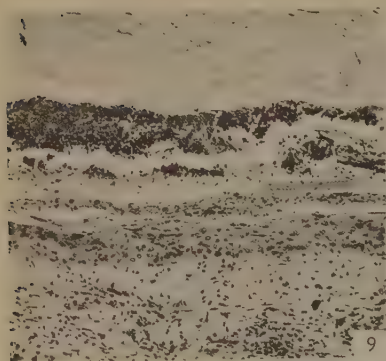
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BILLINGHAM AND SPARROW—STUDIES ON THE NATURE OF IMMUNITY TO
HOMOLOGOUS GRAFTED SKIN



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EXPLANATION OF PLATES

PLATE 1

- Fig. 1. Animal Sr-201. An extensive sheet of well-keratinized epithelium originating from six pure epidermal homografts transplanted 11 days previously. At this stage the epithelium is completely surviving. $\times 0.8$.
- Fig. 2. Animal Sr-121. Transverse section through a biopsy specimen of a pure epidermal homograft of 9 days standing. The epidermis is grossly hyperplastic and overlies an exceedingly rich vascular bed. There is no histological indication of the onset of a specific reaction. $\times 100$.
- Fig. 3. Animal Sr-121. Vertical section through a pure epidermal homograft on the 11th day after transplantation. The hyperplastic epithelium which is now covered by a very thick multi-layered cuticle is completely viable. The differentiating native granulation tissue comprising the graft bed is now becoming infiltrated by lymphocytes whose distribution is still mainly perivascular. $\times 100$.
- Fig. 4. Animal Sr-121. Vertical section through a pure epidermal homograft of 13 days standing. Cellular erosion of the basal layer of the graft epidermis is taking place and the graft bed, which is inflated with oedema fluid, is heavily infiltrated by leucocytes. Breakdown of the homologous epithelial cells is clearly far advanced. $\times 100$.
- Fig. 5. Animal Sr-121. Complete breakdown of the homologous epidermis. This biopsy specimen was removed on the 15th day after transplantation. The necrotic remains of the epithelium are now separating from the bed. Note that the native leucocytes below the graft remnants have nearly all broken down. $\times 100$.
- Fig. 6. Vertical section through a sheet of pure epidermis obtained indirectly by first transplanting a series of sheets of delicate pure epithelium prepared from the skin of its ear to a raw area cut in the skin of the side of a rabbit's chest. A pure epidermal sheet as figured could easily be stripped from the bed 17 days after transplantation. It is completely free from all mesenchymal elements and is mechanically supported by its thick adherent cuticle. $\times 100$.
- Figs. 7, 8. Vertical sections through pure epidermal homografts of 2 and 3 days standing respectively transplanted to specifically immunized animals. Both grafts are intimately united to their beds which are highly vascular. There is only trace survival of the epithelium which is only weakly basiphilic. Note that the stratified cuticular layers of the grafts are grossly distended and blistered by fluid exudates. $\times 100$.

PLATE 2

- Figs. 9, 10. Sections through pure epidermal homografts which have been in residence on specifically immunized animals for a period of 5 days. Note that the degree of epithelial survival is considerably higher than that illustrated in Pl. 1, figs. 7, 8. $\times 100$.
- Figs. 11, 12. Illustrating the 'seeding' test for melanocyte survival in the guinea-pig. Both of the sites illustrated in fig. 11 were 'seeded' with epidermal melanocyte suspensions 17 days previously, while those in fig. 12 were seeded 22 days previously. In each case, one site has received experimentally treated melanocytes, while the other site received the appropriate 'controls'. $\times 1$.
- Fig. 13. Animal Sr-182. The result of the seeding test of viability of Malpighian cells after treatment *in vitro* for 29 hr. at 5°C . with immune serum. The upper site was grafted with experimentally treated cells and the lower site with their controls. Only a trace of epithelium has arisen from the experimentally treated cells; there is an extensive sheet of epithelium on the control site. Photographed on the 13th day after grafting.
- Fig. 14. Animal Sr-225. The very conspicuous difference between the appearance of the areas grafted with Malpighian cells incubated with immune serum and control serum respectively for 23 hr. at 37°C . The lower site was grafted with the experimentally treated cells and the upper with their controls. No trace of epithelium has appeared on the lower site. Photographed on the 13th day after grafting.

THE PASSAGE OF STARCH THROUGH THE STOMACH OF THE SHEEP

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Micro-organisms are now known to be the agents responsible for many important chemical changes in the rumen contents. By the fermentation of polysaccharides they produce large quantities of the lower fatty acids which are absorbed and utilized by the host; they digest a considerable part of the protein in the fodder and resynthesize proteins during their own growth and proliferation; they are capable of converting suitable forms of non-protein nitrogen to protein, and can synthesize many of the vitamins. The accurate assessment of the extent to which such processes take place, however, presents major difficulties—difficulties bound up with the fact that the rumen is a reservoir into which more or less continuous supplies of food, water and saliva enter, and from which some products of the fermentation are absorbed, while others, together with food residues, pass to lower levels of the alimentary tract. As a consequence, the extent to which various compounds are digested, released or synthesized, cannot be determined from the concentrations of these substances in the rumen, and so indirect methods of investigation must usually be applied to such problems.

The present work is concerned with some aspects of the fate of starch during its passage through the compound stomach of the sheep. In the past it was often thought that significant quantities of starch might escape destruction, or alternatively that new starch might be synthesized and stored in the micro-organisms to be passed on to lower levels of the alimentary tract for digestion as in other animals.

EXPERIMENTAL

(1) *Sampling and analytical procedures*

A sheep with permanent fistulae of the rumen and abomasum provided the samples from these two compartments of the living animal; other samples were obtained from slaughtered sheep.

Fistula sheep. The procedure for the establishment of abomasal fistulae has been described by Jarrett (1948), but a larger cannula (internal diameter 10 mm.) was introduced by substituting tubes of increasing sizes over a period of several weeks. In this way sampling of the contents by siphoning was made easy and accurate. Samples from the rumen were taken by means of a metal double-tube sampler—the inner tube slotted and capped at its distal end.

Slaughtered sheep. The abdomen was opened immediately after slaughtering and the organs isolated from one another by tapes tied at the levels of the reticulo-omasal and omaso-abomasal orifices and at the pylorus. The contents were then removed and where necessary smaller samples were selected for analysis.

General treatment of samples. Each sample was weighed, filtered through muslin (about 40 threads/in.) and the solid residues squeezed as dry as possible. These solids were then washed twice with water, the washings being added to the first filtrate. In general, the volume of water used in each washing of the solids from the reticulum, rumen and abomasum was approximately that of the first filtrate. When the concentration of solids was low the volume of wash water was correspondingly reduced. In the case of the omasal contents water was added to give a thin, creamy consistency, and this mixture was then treated similarly to the contents of the other compartments.

The total volume of the combined filtrate was measured and aliquot samples taken for the determination of starch. The sizes of the aliquots were chosen so that the reducing sugars finally determined in the 5 ml. aliquot of hydrolysate (q.v.) would be equivalent to 0.5–2.5 mg. maltose. Further samples were taken for counting the Protozoa, and the solids were reserved for the determination of lignin.

Determination of starch. To the aliquot of the above filtrate three volumes of ethanol were added and the mixture allowed to stand overnight for the complete precipitation of starch and micro-organisms. The precipitate was separated and washed with ethanol by centrifuging, and dried at 70° C. *Preparation of starch extract.* The starch of the precipitate was rendered water-soluble by refluxing for 30 min. with 25 ml. of a mixture of 95 % ethanol containing 1 % by volume of conc. (36 %) HCl solution. After centrifuging while still hot, the precipitate was washed with ethanol, dried at 70° C., and 30 ml. of water added to dissolve the starch.* The mixture was refluxed for 30 min., cooled and the volume adjusted to 50 ml.; it was then centrifuged, and the clear extract used in the hydrolysis of starch. *Hydrolysis of starch.* Aliquots (20 ml.) of the extract were taken for hydrolysis of the starch by the salivary α -amylase method of Hassid, McCready & Rosenfels (1940). After clarification of the digest and the accompanying blank, the volumes were adjusted to 50 ml., centrifuged, and 5 ml. aliquots taken for the determination of the reducing sugars formed. This determination was made by the procedure of Hanes (1936), employing the alkaline ferricyanide method, and expressing the reducing sugars as maltose. Finally, Hassid's value—0.890 mg. maltose per mg. starch—was used to calculate the amount of starch present.

Counting of Protozoa. The ciliate Protozoa were counted in a haemocytometer cell. Formalin was added to stop all movement of the organisms, and whenever necessary their concentration was altered by centrifuging and resuspending in a suitable volume of water. By this means all suspensions counted had approxi-

* The residue after extraction with water showed no iodine-staining reaction, and parallel analyses carried through without the starch being dried from ethanol at 70° C. also indicated that this step did not interfere with solution of the starch in water.

mately the same concentration of Protozoa. In all instances duplicate counts, each of at least 500 Protozoa, were made.

It was established that the distribution given by counts of the Protozoa was Poissonian, and the concentration of the organisms was estimated with a standard deviation of 2.06 %.

Determination of Lignin. The solid residues in the muslin cloth were soaked in ethanol, squeezed free of excess liquid and dried at 40° C. for 48 hr. After several days' equilibration with air the solids were weighed, ground to pass a sieve with holes 0.5 mm. in diameter, and 1 g. samples used for the determination of lignin by the 72 % H₂SO₄ method. The procedure used included a preliminary refluxing for 1 hr. with 5 % H₂SO₄ and the reaction with 72 % H₂SO₄ was allowed to continue for 16 hr. at 16–20° C.

(2) Experiments and results

The distribution of starch in the stomach contents. In the preparation of samples a small part of the starch remains in the solid fraction despite thorough washing. Observations on the distribution of starch between solids and liquid in the rumen and abomasal contents at various times after feeding are given in Table 1.

Table 1. *Distribution of starch in stomach contents of a sheep fed on wheaten hay*

Organ	Sample no.	Time after feeding began (hr.)	Starch (mg./100 g. whole contents)			Starch in solids total starch × 100
			Fluid	Solid residues	Total	
Rumen	1	0	60	10.5	70.5	15
	2	1½	225	40	265	15
	3	4	272	22	300	7
	4	6½	196	24	220	11
Abomasum	1	0	10.9	Too small for analysis	—	—
	2	1½	21.0	3.2	24.4	13
	3	4	68.0	8.9	76.8	12
	4	6½	46.5	7.7	54.2	14

The distribution of starch between bacteria and ciliate Protozoa in the rumen contents. Rumen contents were sampled and separated into liquid and solid portions as described. The first filtrate was kept separate from the washings and both were gently centrifuged. The point at which all the Protozoa but a minimum of bacteria were thrown down was decided by microscopic examinations of the supernatant fluid. The various fractions were then examined for their starch content. The numbers of ciliate Protozoa in the first filtrate and in the residual fluid contained in the washings were also determined. The data are summarized in Table 2.

The stability of starch in the abomasum. Four samples of abomasal material containing starch were collected. Starch was determined in two of them immediately, and in the others after incubation at 39–40° C. for 1 and 3 hr. respec-

tively. There was no significant change in the starch content of the incubated samples.

Starch and lignin in the rumen and abomasum of a sheep fed on diets of varying starch content. Six experiments were carried out, and three types of fodder were employed to give starch concentrations ranging from 0.4 to 18.5% of the weight of fodder eaten. Lucerne hay provided the low-starch diet, various samples of

Table 2. *Distribution of starch between bacteria and Protozoa in the fluid portion of a sample of rumen contents*

	Weight (g.)	No. of ciliate Protozoa	Starch (mg.)	
			Protozoal sediment	Supernatant
First filtrate	221	115×10^6	573	139
Residual liquid*	35	13.6×10^6	90	70

* Calculated as difference between weight of sample of rumen contents and the combined weights of first filtrate and residual solids.

Table 3. *Starch and lignin in the rumen and abomasum*

Fodder* 800 g. Wheaten hay: Starch 5.3%, 42 g. fed each day.
Lignin 6.9%, 55 g. fed each day.

Time of sampling	Rumen contents			Abomasal contents		
	Starch (g./kg.)	Lignin (g./kg.)	Ratio Starch/Lignin	Starch (g./kg.)	Lignin (g./kg.)	Ratio Starch/Lignin
9.30 a.m.	0.524	8.85	0.059	0.092	3.81	0.024
10.30	2.34	6.62	0.354	0.099	4.00	0.025
11.30	2.88	4.11	0.700	0.150	1.25	0.120
12.30 p.m.	2.82	4.18	0.675	0.208	1.25	0.166
1.45	3.20	3.69	0.875	0.425	1.53	0.278
3.00	3.34	5.08	0.656	0.574	8.62	0.067
4.40	2.59	5.69	0.455	0.673	6.80	0.099
7.10	2.14	5.76	0.372	0.622	6.25	0.100
10.40	1.40	6.42	0.218	0.563	10.82	0.052
6.30 a.m.	0.702	8.87	0.079	0.236	5.87	0.040
9.30	0.403	10.38	0.039	0.127	3.68	0.034

* Feeding started at 9.30 a.m. immediately after the first samples were taken.

wheaten hay gave intermediate concentrations, and a mixture of wheaten hay with dehydrated potato chips was used for the high-starch fodder. Feeding of each diet was continued for at least 2 weeks before sampling from the stomach. The feeding period was restricted to 3 hr. in the morning in the first five experiments, and the sheep consumed all of the food offered. Water was available at all times. Samples from the rumen in three experiments, and from the abomasum in all of them, were collected at frequent intervals throughout the 24 hr. and subjected to analysis for starch and lignin. The complete data for one experiment are given in Table 3

and Fig. 1. A summary of the relevant findings in five of the experiments is given in Table 4 to show the amounts of starch calculated to pass through the abomasum in 24 hr.* In Table 5 starch/lignin ratios in the rumen and abomasum are compared.

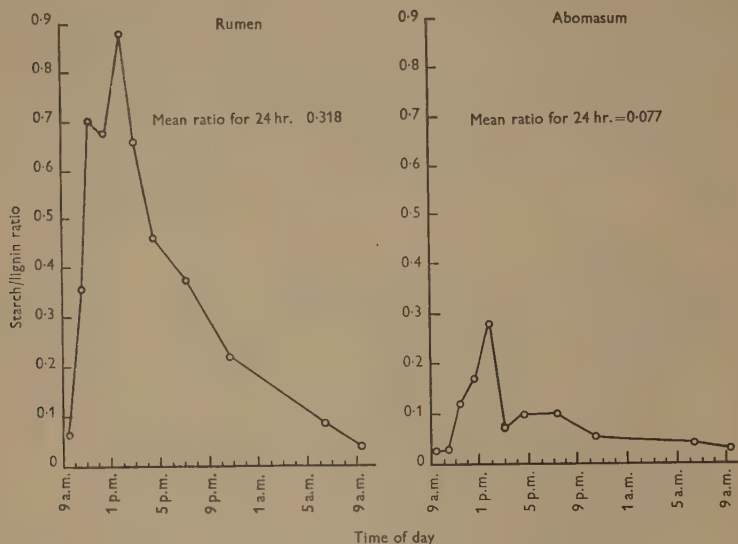


Fig. 1. Starch/lignin ratios in the rumen and abomasum.

Table 4. Starch passing through the abomasum in 24 hr.

No.	Fodder	Starch fed (g.)	Mean ratio Starch Lignin during 24 hr. in abomasum*	Lignin fed (g.)	Starch passing through abomasum in 24 hr. (g.)
1	Lucerne hay, 800 g.	2.9	0.0152	71	1.1
2	Wheaten hay, 800 g.	20.9	0.0339	63	2.1
3	Wheaten hay, 600 g.	41	0.0865	41	3.6
4	Wheaten hay, 800 g.	42	0.077	55	4.2
5	Wheaten hay 600 g., potatoes 200 g.	14.8	0.151	51	7.8

* Computed from graphs as in Fig. 1.

Starch and Protozoa in the stomachs of slaughtered sheep. The results of the determination of starch and Protozoa in the rumen, reticulum and omasum of slaughtered sheep are given in Table 6. All of these animals were fed on wheaten hay prior to being slaughtered.

* In experiment no. 6 the animal was fed ad lib. The amount of lignin consumed was not determined, and the data therefore could not be used for calculation of the amount of starch reaching the abomasum.

Table 5. *Starch/lignin ratios in the rumen and abomasum*

Exp. no.	Mean ratio starch/lignin	
	Rumen	Abomasum
4	0.318	0.077
5	0.439	0.151
6	0.370	0.053

Table 6. *Starch and Protozoa in the fluid portion of the contents of the rumen, reticulum and omasum of slaughtered sheep*

Sheep	Organ	Ciliate Protozoa (numbers/100 ml. fluid)	Starch (mg./100 ml. fluid)
1	Rumen	44×10^6	51
	Reticulum	58×10^6	54
	Omasum	23×10^6	29
2	Rumen	46×10^6	136
	Reticulum	50×10^6	162
	Omasum	8×10^6	33
3	Rumen	48×10^6	35
	Reticulum	48×10^6	26
	Omasum	10×10^6	13
4	Rumen	42×10^6	140
	Reticulum	41×10^6	72
	Omasum	12×10^6	59

DISCUSSION

The constitution of the starch or starch-like polysaccharides in the stomach contents of ruminants has not yet been established. There can be little doubt that it varies from time to time, since there may be present a variety of plant starches, as well as the starch synthesized by the micro-organisms. Numerous references have been made to the iodine-staining polysaccharides formed in the micro-organisms of the rumen, but until recently no detailed examination of them has been attempted. Masson & Oxford (1951) have isolated such material from rumen holotrichous ciliates fed on glucose and have produced evidence in favour of regarding the polysaccharide as starch rather than glycogen; oligotrichous ciliates were also examined. But it was concluded that the final decision as to the constitution of the polysaccharides must await further studies.

The nature of the polysaccharides present in the bacteria of the rumen has received less attention. It may be noted that the polysaccharide formed in *Clostridium butyricum* from sucrose has been shown to be of the amylopectin type (Hobson & Nasr, 1951). This organism is known to be present in the caecum of the pig and may occur in the rumen. But it may not in any way be representative of the bacterial population of the rumen.

In the present work, starch has been measured by a method developed for its determination in certain plant materials known to contain approximately 80%

amylopectin and 20% amylose; and in so far as the mixed starches in the stomach contents may contain a different proportion of amylopectin (or yield different amounts of reducing sugars under the influence of salivary α -amylase), so the conversion factor for calculating starch from these reducing sugars may need modification.

It is stressed that the meaning of the term 'Starch' as used in the present work must be qualified in this way. With this proviso there is no need to avoid the use of the term in dealing with the starches of unknown constitution in the stomach of the sheep.

(1) *Distribution of starch in the stomach contents.* The observations summarized in Table 1 indicate that by far the larger part of the starch is to be found in the fluid portion of the contents. Only 7-15% remained in the solids after they had been washed free of the residual fluid with water. This small residue may have been lodged in bacteria closely attached to the plant tissues, or in the plant material itself.

It is, of course, already realized that both the amount of starch in the rumen and its distribution between Protozoa and bacteria may vary widely with the nature of the fodder and the time after feeding. The examination of a sample of rumen contents drawn within a few hours of feeding a sheep with wheaten hay (Table 2) suggests that a very considerable part of the starch may be present in the ciliate Protozoa.

Unfortunately, it is not possible to separate the Protozoa completely from the bacteria by centrifuging. Nevertheless, the partition of starch between ciliate Protozoa and bacteria in this particular sample can be determined if it is assumed that the same proportions of bacterial starch were spun down from the first filtrate and from the residual liquid (in the form of washings).

If there were S mg. of protozoal starch in the sediment of the first filtrate the distribution of starch could be written:

	First filtrate	Residual liquid
Sediment: Protozoal starch	S	$13.6S/115$
Bacterial starch	$573-S$	$90-13.6S/115$
Supernatant: Bacterial starch*	139	70

And on the assumption made above

$$(573-S) : 139 = (90 - 13.6S/115) : 70.$$

Therefore protozoal starch = $S + 13.6S/115 = 576$ mg. = 66% of the total starch.

The calculation is made possible by the fact that the proportion of Protozoa to bacteria is different in the first filtrate and in the residual fluid. Although it is unlikely that the assumption on which it is based is strictly true, the result may be of interest as an approximation. It should be noted that the numbers of Protozoa found in sheep fed on wheaten hay are usually much smaller than those found in pasture-fed animals.

(2) *The stability of starch in the abomasum.* It has already been indicated by Heald (1951) that starch is not degraded in the abomasum. This was confirmed in the present work by the fact that no change was found in the starch content of abomasal material incubated at 39-40° C. for 1 or 3 hr.

* Separate tests showed no starch remaining after centrifuging at $17,000 \times g$.

(3) *The amount of starch reaching the abomasum.* Heald (1951) estimated that in 24 hr. about 5 g. of 'glucose-containing carbohydrate' (referred to also as 'combined-glucose' and approximately equivalent to starch) was present in the micro-organisms of the digesta passing through the abomasum of a sheep fed on chopped hay (concentration of starch not stated). The estimation was made from the observed concentration of 'combined-glucose' in the abomasal contents, and the assumed passage of 400 ml. of the contents through the organ in 1 hr.—an assumption based on the results of the experiments of Phillipson, Green, Reid & Vowles (1949). While the result can be considered to be in close agreement with the present findings, it gives no indication of the relationship between the amount of starch fed and the amount reaching the small intestine. In the present work this relationship was examined, and at the same time an approach was used which allowed all the data for any one computation to be derived from the same animal. Because lignin as determined in these experiments has been shown to be an undigested fraction of the fodder (Gray, 1947; see also Kane, Jacobson & Moore, 1950), the amount passing through the abomasum in 24 hr. must be equal, within small limits of variation, to the amount eaten, provided the animal consumes each day a constant weight of a fodder of constant composition. If, under such circumstances, the concentrations of starch and lignin in the abomasal contents are measured at sufficiently frequent intervals, it is possible to compute the amount of starch passing through the organ from the mean ratio of starch to lignin during the day. Table 4 summarizes the results obtained from five experiments in which these measurements were made. It can be seen that an increase in the amount of starch fed from 3 to 150 g. per day led to only a small increase (from 1 up to 8 g.) in starch passing through the abomasum. It is clear from these results that the passage of starch to the small intestine is not sufficient to be an important nutritional factor in sheep, even when diets high in starch are being fed.

(4) *The passage of starch from the rumen to the abomasum.* Heald (1951) found that the concentrations of 'combined glucose' in the micro-organisms of the rumen and abomasum were much the same, and on an assumption that the flow of digesta out of the rumen was equal in quantity to the flow out of the abomasum, he concluded that the same amount of starch passed out of each organ. The assumption on which this conclusion is based has no experimental proof; moreover, in the present experiments it was found that the concentration of starch was much higher in the rumen than in the abomasal contents, and the same was true of the starch-lignin ratios in the two organs (Table 5). It might be expected that such a difference in the ratios would occur for a short time after feeding if the diet contained much starch. But, in fact, the difference, though most marked immediately after feeding and least at the end of the feeding cycle, remained significant throughout the greater part of the day. Since starch is not broken down in the abomasum it must be concluded that it is partly destroyed in the omasum, or else does not pass through the omasum at the same rate as lignin. Convincing evidence for its destruction in the omasum is provided by the data obtained from slaughtered sheep (Table 6) which show that the concentrations of both Protozoa and starch

are very much reduced in the fluid portion of the omasal contents. It seems that such a reduction could only come about through destruction of some of the micro-organisms and their content of starch. In fact, the destruction may be even more extensive than these changes in concentration suggest, because there is evidence (Garton, 1951) that a considerable amount of water may be absorbed through the walls of the omasum.

Finally, microscopic inspection of omasal contents shows that most of the larger Protozoa there are no longer motile, and that many distorted and broken organisms are present. Similar observations have apparently been made elsewhere (Cuthbertson, 1952).

SUMMARY

1. The greater part of the starch in the stomach of the sheep is present in the fluid portion of the contents, and a large proportion of it may be carried in the ciliate Protozoa of the rumen.

2. Starch:lignin ratios in the stomach contents suggest that there may be an extensive destruction of starch in the omasum, and this is strongly supported by the lowered concentrations of both starch and Protozoa in the omasal liquid.

3. A maximum of only 7.8 g. of starch was calculated to pass through the abomasum daily when the ration contained 148 g. starch. When 20-40 g. of starch were fed the quantity passing through was about one-tenth of the quantity ingested, and when the ration contained only 2.9 g. starch, 1.1 g. reached the abomasum.

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FUNCTIONS OF THE OMASUM IN THE STOMACH OF THE SHEEP

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The processes which occur in the omasum of the ruminant have long been a subject of speculation and debate (Favilli, 1937). It was at first thought that the fluid portion of the digesta passed on from the reticulum was squeezed out in the omasum, and the larger food particles comminuted there, but no evidence has been offered which proves that either of these processes actually take place. As an alternative explanation of the characteristically high concentration of solids in the omasum it was later suggested that large amounts of water might be absorbed through the walls of the organ, and this suggestion has recently received strong support from the observation (Garton, 1951) that the concentrations of certain inorganic ions are often much higher in the omasal fluid than in the rumen fluid. Nevertheless, the possibility of permeation of the omasal wall by these ions makes them unreliable as accurate markers of the absorption of water; and the further possibility that some of the reticular contents may bypass the omasum and flow directly to the abomasum via the omasal groove has rendered equivocal the use of markers for the assessment of the extent to which water is absorbed. It has been suggested (Elsden, Hitchcock, Marshall & Phillipson, 1946) that the greater part of the fatty acids in solution in the liquid portion of the material received from the reticulum are absorbed from the omasum, but here again the evidence is incomplete because of lack of knowledge of how liquids and solids are passed through the omasum. Fatty acids are undoubtedly absorbed there (Barcroft, McAnally & Phillipson, 1944), and the ratio of acids to solids is much reduced (Elsden *et al.* 1946), yet if fluid were separated from the solids mechanically, rather than absorbed through the walls of the omasum, the absorption of fatty acid need not be as extensive as has been implied.

More recently it has been reported (Weller & Gray, 1954) that breakdown of part of the starch, and destruction of many of the ciliate Protozoa which enter the organ from the reticulum, are effected in the omasum.

It remains to be shown, in the first place, whether any fluid is squeezed away from the digesta in the omasum, and in the second place, whether significant amounts of reticular contents pass directly to the abomasum. It must be stressed that only when this information is available can an attempt be made to measure the extent to which water and fatty acids are absorbed from the ingesta after they have passed through the reticulo-omasal orifice to the omasum.

EXPERIMENTAL

The concentrations of volatile fatty acid, starch, nitrogen, solids and lignin were measured in the contents of the various compartments of the stomachs of sheep which had been slaughtered after having fed for at least 1 week on chaffed wheaten hay. The procedures used for the collection of samples and their separation into fluid and solid fractions, and the chemical methods employed in the determinations of lignin, starch and fatty acids have been described in earlier publications (Weller & Gray, 1954; Gray, Pilgrim & Weller, 1950). Nitrogen determinations were made by the Kjeldahl method on both the solid and liquid portions of the stomach contents. In most instances the liquid portion was separated into micro-organisms and residual liquid by centrifuging at 15,000 r.p.m. ($17,000 \times g$) for 30 min. and nitrogen determinations were made on both fractions. Data concerning the solids, lignin and nitrogen in the rumen, reticulum and omasum of seven slaughtered sheep are given in Table 1, the concentrations of lignin and starch in all the stomach compartments of six sheep are given in Table 2, and similar data for the fatty acids in Table 3.

DISCUSSION

One object of the studies reported here was to obtain an estimate of the extent to which water is absorbed from the omasum. It might seem at first sight that this could be accomplished by measuring the concentrations of a suitable marker in the fluid portion of the contents of the reticulum and of the omasum, and considering the increase in its concentration in the omasal fluid to be a reflexion of the extent to which water had been absorbed from that organ. But there are two equally important considerations to be taken into account before this can be done. The first is that some of the material passing over from the reticulum may reach the abomasum, via the omasal groove, without entering between the leaves of the omasum. The other is that liquid may be squeezed away mechanically from the solids, and so reach the abomasum ahead of them. Thus only a fraction of the liquid may be represented in the omasal fluid examined. The extent to which these phenomena occur must be known before a reliable estimation of water absorption could be made by use of a marker in the stomach fluids.

Examination of the ratios of nitrogen to lignin in the rumen, reticulum and omasum (Table 1) shows that separation of liquid from the solids by squeezing of the digesta in the omasum cannot be at all extensive. A large part—more than 50%—of the total nitrogen of the stomach contents is present in the liquid portion, and therefore, if liquid were squeezed either ahead of the solids, or back into the reticulum, the ratio of nitrogen to lignin would be lowered in the omasal contents. In fact, the data show that this ratio is usually as high in the omasum as in the reticulum or rumen. Even greater changes would be brought about in the ratio of nitrogen in the liquid portion to lignin in the whole contents, but this ratio again is no lower in the omasum than in the first two compartments. It is clear that mechanical squeezing away of liquid can contribute very little to the increase in concentration of the residual plant solids in the omasum.

Table 1. Nitrogen and lignin in the stomachs of slaughtered sheep

Sheep no.	Hours after the end of feeding	Organ	Solids (g./100 g. contents)	Lignin (g./100 g. contents)	Nitrogen (mg./100 g. contents)				Ratio of mg. nitrogen to g. lignin in whole contents
					Plant residues	Micro-organisms*	Residual liquid	Total contents	
1	2½	Rumen	9.3	1.48	72	92	28	192	81
		Reticulum	11.6	1.91	84	125	31	240	82
		Omasum	16.4	2.71	135	224	48	407	100
2	3	Rumen	8.9	1.50	64	98	25	187	82
		Reticulum	6.0	0.95	50	81	24	155	111
		Omasum	13.7	2.40	122	203	34	359	99
3	4	Rumen	10.0	1.43	88	112	12	212	87
		Reticulum	6.6	0.94	62	92	12	166	111
		Omasum	16.0	2.40	158	224	18	400	101
4	4	Rumen	11.2	1.65	103	109	12	224	73
		Reticulum	9.2	1.36	86	79	13	178	68
		Omasum	17.0	2.60	163	166	16	345	70
5	4	Rumen	12.1	1.75	132	121	14	267	77
		Reticulum	10.0	1.47	118	113	15	246	87
		Omasum	16.6	2.70	146	224	23	393	91
6	8	Rumen	8.5	1.29	87	109	15	211	96
		Reticulum	7.0	1.07	72	91	13	176	97
		Omasum	15.4	2.43	162	213	18	393	95
7	16	Rumen	8.0	1.26	64	96	17	177	100
		Reticulum	10.0	1.49	75	102	17	194	80
		Omasum	17.3	2.36	130	149	19	298	71

* A similar sample of micro-organisms contained, on a dry-weight basis, 1.1 % crude fibre, 1.6 % lignin, and 54.0 % crude protein.

If some reticular material were able to pass directly to the abomasum without entering the main body of the omasum, then the abomasum would receive a mixture of reticular and omasal contents. Now the ratio of starch to lignin is much higher in the reticulum than in the omasum (Table 2), and any mixture of contents from the two organs would have a starch/lignin ratio of intermediate value. With one exception, which is considered to be an abnormal instance, the data in Table 2

Table 2. *Starch and lignin in the stomachs of slaughtered sheep*

Sheep no.	Hours after the end of feeding	Organ	Starch (g./100 g. contents)	Lignin (g./100 g. contents)	Ratio Starch/Lignin $\times 1000$
1	2½	Rumen	0.046	1.48	31
		Reticulum	0.048	1.91	25
		Omasum	0.024	2.72	9
		Abomasum	0.010	0.99	10
3	4	Rumen	0.126	1.43	88
		Reticulum	0.067	0.94	71
		Omasum	0.050	2.40	21
		Abomasum	0.025	1.23	20
4	4	Rumen	0.067	1.65	41
		Reticulum	0.046	1.36	34
		Omasum	0.028	2.60	11
		Abomasum	0.020	1.12	18
5	4	Rumen	0.122	1.75	70
		Reticulum	0.113	1.47	77
		Omasum	0.062	2.70	23
		Abomasum	0.031	1.58	20
6	8	Rumen	0.043	1.29	33
		Reticulum	0.029	1.07	27
		Omasum	0.022	2.43	9
		Abomasum	0.016	1.64	10
7	16	Rumen	0.013	1.26	10
		Reticulum	0.010	1.49	7
		Omasum	0.008	2.36	3
		Abomasum	0.003	0.90	3

show that the ratio is very nearly the same in the abomasum as in the omasum; and because there is no destruction of starch in the abomasum (Heald, 1951; Weller & Gray, 1954), it must be concluded that only small amounts of reticular contents can pass directly to the abomasum. Since most of the starch is present in the micro-organisms suspended in the liquid portion of the digesta (Weller & Gray, 1954) the fact that the omasal and abomasal contents have the same starch/lignin ratio is a further indication that there is no squeezing of omasal liquid ahead of the solids.

When sheep no. 4 was slaughtered it became apparent at once that the contents of the abomasum were abnormal in that there were very many coarse particles of fodder present. All of the other animals of the series, and many examined at other times, exhibited extremely few coarse particles in the abomasum. It seemed obvious from the appearance of the stomach contents that a large quantity of reticular material had passed directly to the abomasum, and this was borne out by

the fact that the starch/lignin ratio in the abomasum was intermediate in value between the ratio in the reticulum and that in the omasum. It was concluded that in this particular sheep, reticular contents passed directly to the abomasum at the time of slaughtering, either through abnormal movements of the gut or through manipulation of the organs during their separation. The fact that coarse particles of fodder are normally absent or rare in the abomasum is, of course, itself an indication of the absence of any direct passage of reticular contents to the abomasum.

Table 3. *Volatile fatty acids and lignin in the stomachs of slaughtered sheep*

Sheep no.	Hours after the end of feeding	Organ	Fatty acid (ml. N/100/100 g. contents)	Lignin (g./100 g. contents)	Ratio $\frac{\text{Fatty acid}}{\text{Lignin}}$	Fatty acid absorbed in omasum (%)	Concentration fatty acid in omasum as % of conc. in reticulum (%)
1	2½	Rumen	494	1.48	334	40	86
		Reticulum	402	1.91	211		
		Omasum	346	2.72	127		
		Abomasum	89	0.99	90		
2	3	Rumen	380	1.50	253	69	78
		Reticulum	356	0.95	375		
		Omasum	277	2.40	115		
		Abomasum	—	—	—		
3	4	Rumen	531	1.43	372	69	80
		Reticulum	525	0.94	558		
		Omasum	419	2.40	174		
		Abomasum	75	1.23	61		
4	4	Rumen	515	1.65	312	64	70
		Reticulum	466	1.36	343		
		Omasum	326	2.60	125		
		Abomasum	73	1.12	65		
5	4	Rumen	602	1.75	344	56	81
		Reticulum	536	1.47	365		
		Omasum	433	2.70	161		
		Abomasum	81	1.58	51		
6	8	Rumen	508	1.29	394	67	75
		Reticulum	466	1.07	436		
		Omasum	352	2.43	145		
		Abomasum	70	1.64	43		
7	16	Rumen	418	1.26	332	50	79
		Reticulum	400	1.49	268		
		Omasum	316	2.36	134		
		Abomasum	42	0.90	47		

Since these observations show that liquid is not squeezed away mechanically from the solids in the omasum and that reticular material does not bypass the omasum, it is possible to determine the amount of water absorbed from the omasum by the increase in concentration of any suitable marker in the omasal contents. Lignin, as determined in these experiments, affords a more acceptable marker than the solids of the stomach contents because it is known to be an undigestible fraction of them (Weller & Gray, 1954). Nevertheless, the use of the solids themselves as marker would lead to much the same result, since com-

paratively little further digestion takes place after they leave the reticulum. The increased concentration of lignin in the omasum in the present series of experiments shows that 33–64% of the water present in the reticular contents was absorbed through the walls of the omasum during the passage of the contents through that organ.

Turning now to consideration of the fatty acids, it is clear from Table 3 that their concentration in the omasal fluid is usually about 20% lower than their concentration in the rumen or reticular fluids. Since a considerable part of the water entering the omasum is absorbed through the omasal walls, it follows that an even greater proportion of the fatty acid is absorbed there also. The extent of absorption calculated from the fatty acid/lignin ratios in sheep 1–7 ranges from 40 to 69% of the acid reaching the omasum from the reticulum.

Examination of the solids and fatty acid concentrations, and the fatty acid/lignin ratios in the rumen and reticulum shows that between these two organs there must be some differential movement of solids and liquid. While the concentration of fatty acid is mostly somewhat higher in the rumen, the fatty acid/lignin ratio is higher in the reticulum—a natural consequence of the lower concentration of solids in that organ. (In sheep 1 and 7, however, there was a higher concentration of solids in the reticulum, and a corresponding lower fatty acid/lignin ratio.) The present observations have shown that such differential movements of solids and liquid do not occur in the omasum; the lowered fatty acid/lignin ratio in the omasum therefore gives a measure of the fatty acids absorbed from the digesta after they leave the reticulum.

It is important to note that comparisons between the contents of the various compartments of the stomach should not be made too soon after feeding, because the omasal and abomasal contents immediately after the animal has fed would probably be representative of conditions which obtain only at the end of the feeding cycle, while the rumen and reticulum would contain a very large proportion of fresh fodder.

From the above considerations and from observations reported earlier, it may be concluded that practically all of the digesta are passed from the reticulum through the body of the omasum where they lose very considerable quantities of both water and fatty acids by absorption. While the digesta are in the omasum a large proportion of the ciliate Protozoa that have developed in the rumen contents are destroyed and the starch granules contained within are released and degraded. There is no evidence that fluid is squeezed away mechanically as the digesta pass through the omasum; the greatly increased concentration of solids are due, rather, to the absorption of water through the large surfaces presented by the leaves of the organ. There is a marked difference in the degree of comminution of the plant residues in the reticulo-rumen and in the omasum, but actual proof of the breaking up of the large particles by mechanical action of the leaves of the omasum is wanting.

SUMMARY

1. Starch/lignin ratios and nitrogen/lignin ratios in the various compartments of the stomachs of slaughtered sheep indicate that only very small amounts of reticular contents may pass directly to the abomasum via the omasal groove, and that little if any liquid is squeezed away mechanically from the solids in the omasum.

2. The amount of water calculated to be absorbed from digesta entering the omasum ranged from 33 to 64% and the amount of fatty acid from 40 to 69% in seven sheep slaughtered for these estimations.

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THE EFFECTS OF HIGH HYDROSTATIC PRESSURES ON A SUCTORIAN

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(With Plates 3-5)

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INTRODUCTION

High pressure tends to encourage changes which result in a decrease of volume. It may affect the packing and configuration of molecules, with resulting changes in the properties of the materials concerned, and it may influence the rate and equilibrium of chemical reactions if a volume change is involved. Clearly the effects of pressure on a living cell may be very complicated.

At biological temperatures water is compressed by about 5% at 1000 atm. (about 15,000 lb./sq.in.), and its viscosity is increased by about 5%. Lipoid components of protoplasm no doubt undergo changes in the same direction, and might also solidify. Myosin gels solate progressively with increasing pressure in experiments ranging up to 8000 lb./sq.in. (544 atm.) (Marsland & Brown, 1942), and the same appears to be true of the plasmagel of living cells (Brown, 1934; Brown & Marsland, 1936). The rounding up of *Amoeba* (Marsland & Brown, 1936), and the disappearance of cleavage furrows in dividing sea-urchin eggs (Marsland, 1938) at these pressures, are attributed to the solation of the cortical gel. Moderately high pressure also increases the tension exerted by contracting muscle (Brown, 1934, 1936). The effects of high pressure on proteins no doubt play an important part in these phenomena. Egg albumen is coagulated at 5000-7000 atm. (Bridgman, 1914), and various enzymes are reversibly or irreversibly inactivated at comparable pressures (Curl & Jansen, 1950*a, b*). It is therefore possible that less drastic effects on molecular structure occur at lower pressures. Under certain experimental conditions and particularly at rather high temperatures, catalysis by various enzymes is accelerated by pressures of around 4000-7000 lb./sq.in. (272-476 atm.) (Johnson, Brown & Marsland, 1942*a*; Eyring, Johnson & Gensler, 1946; Werbin & McLaren, 1951). It has been suggested that high pressure prevents the unfolding of proteins from the globular state and so opposes denaturation. Narcosis by certain drugs is counteracted by high pressure, presumably because of some interaction between drug and enzyme (Johnson, Brown & Marsland, 1942*b*). It is not clear from all this work to what extent in living cells high pressure acts merely by reason of changes in volume of enzyme and substrate during activation and reaction, and to what extent it actually alters the structure of enzymes or substrate and so brings these into greater or less steric conformity, in accordance with Goldacre's (1952) model. Although work has so far been centred on the effects of pressure on enzyme systems,

there are other interesting fields open for investigation, including ionization and the configuration of molecules at interfaces. The biophysical chemistry of moderately high pressures is still in a very early state.

On the biological side the need for more detailed observations on a wider variety of material has led to a new design of pressure vessel for use with the microscope. With this an initial investigation has been made of the effects of pressures ranging up to 15,000 lb./sq.in., or just over 1000 atm., on the fresh-water suctorian *Discophrya piriformis* Guilcher (1947).^{*} This animal feeds on holotrich ciliates, the cytoplasm of which it appears to suck up through its tentacles. During the process of feeding the body surface expands, and there is evidence to suggest that this expansion is an active process which serves to lower the internal pressure of the organism (Kitching, 1952*a*). It will be shown in this paper that hydrostatic pressures of over 2000 lb./sq.in. cause an expansion of the pellicle, and sometimes of the protoplasmic surface as well, perhaps similar to the expansion which occurs during feeding. At rather higher pressures results are obtained which provide a basis for comparison with amoebae and dividing sea-urchin eggs. The course of recovery after release of pressure throws interesting light on the relation of protoplasm and pellicle.

METHODS

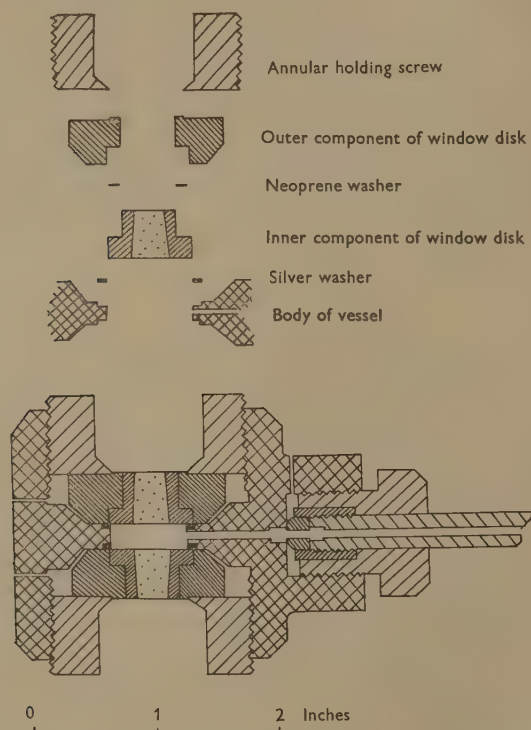
Discophrya was cultured in Bristol tap water in covered glass dishes, and was fed periodically with *Paramecium*. In some cases silk threads were floated on the surface of the water as a convenient substrate.

The pressure vessel, designed by Mr G. A. Shephard, Alkali Division, Imperial Chemical Industries Ltd., is shown in Text-fig. 1. It consists of a 'high tensile' steel box with two glass windows set in steel disks which are held in position by means of annular holding screws. The upper disk with its window is removed when material is to be mounted in the pressure vessel. (Each window disk actually consists of two components. Details of the window assembly are also shown in Text-fig. 1. The neoprene washer protects the window from stress due to the holding screw. The two components of the window disk are only separated when it is necessary to fit a new glass. Each holding screw is slotted to receive a key with which it can be tightened or undone, but this is not shown in Text-fig. 1.) The pressure vessel is connected by heavy-walled steel tubing to a hydraulic pump fitted with a pressure gauge.

The pressure vessel is held in a vice, and is used in conjunction with a suitably modified microscope. A Watson 'Holos' objective ($\times 10$, 25 mm.) has been used in this work, together with an inclined extension tube fitted with a screw microscope eyepiece having a magnification of $\times 25$. Photographs were taken with a Leica microscope camera with a $\times 20$ eyepiece. On the advice of Dr C. R. Burch, a plano-convex lens specially made by him, with its centre of curvature coinciding

^{*} This species is closely related to *Podophrya collini* Root (1915), and was called *Podophrya* sp. in my earlier papers (Kitching, 1951, 1952*a, b*). It reproduces by evaginating a single internally produced embryo, and therefore in accordance with Guilcher's (1950) classification must be called *Discophrya*.

with the position of the organism under observation, was placed in oil-immersion contact with the outer surface of the upper window, to correct for spherical aberration. The 'Holos' objective (N.A. = 0.30) will have a maximum numerical aperture of 0.45 when used in conjunction with this oil-immersion correcting lens. The cone of light diverging from the organism in the hanging drop is restricted by the geometry of the window of the pressure vessel, which has a maximum numerical aperture of 0.58. The maximum numerical aperture for the condenser, illuminating



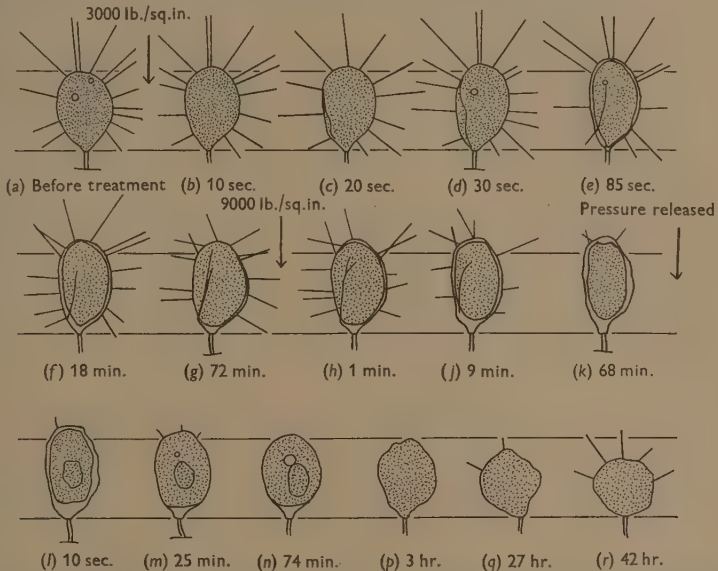
Text-fig. 1. Diagram of pressure vessel. The window assembly and method of setting up window are shown above. Glass parts are dotted.

the organism through the lower window and through the cavity of the pressure vessel (containing medicinal paraffin) is about 0.40. In practice the window glass sometimes developed slight internal cracks, and the resolution suffered somewhat as a result. The photographs (Pls. 3-5) show that it was adequate for the purpose. The centre of the field was used, and chromatic errors were not obtrusive.

The pressure vessel was filled with medicinal paraffin ('mineral oil'). Experimental material was mounted in medium from its own culture in a hanging drop on

the inner side of the upper window. After a preliminary examination on the stage of an ordinary microscope, the window in its disk was set up in the pressure vessel. The *Discophrya* chosen for observation lay with its long axis in one optical plane.

Discophrya was observed before compression, during exposure to one or several different hydrostatic pressures, and finally after release of pressure. Pressures of from 1000 to 15,000 lb./sq.in. (68–1020 atm.) were used, and the periods of application ranged from 5 min. to 8 hr. The pressure was raised in from 1 to 3 sec., and released in less than that time. Sometimes *Discophrya* which had survived exposure to high pressure was left in the pressure vessel for several days after the pressure had been released. It remained alive and apparently healthy; there were no signs of deterioration due to confinement in the pressure vessel.



Text-fig. 2. Diagrammatic tracings from photographs of a newly settled *D. piriformis*. The pressure was raised to 3000 lb./sq.in. for 73 min., and then to 9000 lb./sq.in. for 72 min. See the text for a fuller account.

RESULTS

Effects of high pressure on the general appearance

For convenience in description an account will first be given of the single experiment on a newly settled *Discophrya* illustrated in Text-fig. 2. This figure consists of diagrammatic tracings of a few of the photographs taken during this experiment. About 20 sec. after application of 3000 lb./sq.in., a fold or crease became visible in the body surface (Text-fig. 2, c), and it deepened during the next minute (d, e). The protoplasm also became separated by a narrow space from the overlying pellicle. After a period of 73 min. at 3000 lb./sq.in. the pressure was raised to 9000 lb./sq.in.

for 72 min. The protoplasm gradually became further separated from the pellicle, and eventually appeared to occupy a smaller volume (Text-fig. 2, *k*) than originally. Most of the tentacles disappeared, and there was no detectable activity of the contractile vacuole at this pressure. On release of pressure the meganucleus became immediately visible (*l*), and the protoplasm gradually filled up the pellicle (*m*). The contractile vacuole first became visible about 25 min. after the release of pressure, and was active from then onwards. The few remaining short tentacles disappeared, the body surface became somewhat irregular, the meganucleus faded (*p*), and after many hours, during which there was a further reorganization of the shape of the body, tentacles reappeared (*q*, *r*).

The following summary is based on thirty-two experiments in which photographs were taken at frequent intervals as a record of changes in the shape of the body, and on a number of other experiments without photographs. Sometimes several organisms were kept under observation in a single experiment. In most experiments pressure was applied for 10–60 min., but in some for a much longer time.

1000 lb./sq.in. (68 atm.). No effects on the shape or size of the body were observed with the pressure maintained for 60 min. (in eight individuals) or less.

2000 lb./sq.in. (136 atm.). Creasing appeared some 15 sec. to 1 min. after the application of pressure and usually attained its maximum within about 2 min. It might occur anywhere on the surface of the body and in any direction. The creasing subsequently became reduced, and (in six individuals out of nine) the body surface became completely smooth and rounded again within an hour, even though the pressure was maintained. In some cases, as a stage in the disappearance of the wrinkles, the pellicle became minutely corrugated (see Kitching, 1954, fig. 1). The tentacles remained normal in appearance. No further changes occurred on release of the pressure.

3000 lb./sq.in. (204 atm.). The body became creased as at 2000 lb./sq.in., but the creasing persisted as long as the pressure was maintained. After about 10–15 min. the protoplasm in some cases became partly separated from the pellicle. The pellicle appeared from a comparison of photographs to be larger than it had been before the application of pressure, but this question will be considered again later (p. 61). In the shorter experiments the tentacles appeared unaffected, and this is true also of a *Discophrya* which was maintained at 3000 lb./sq.in. for 3 hr., the body being heavily wrinkled. In another case after 8 hr. at this pressure the tentacles were reduced in number and abnormally short. On release of pressure the protoplasm spread back to the pellicle within a few minutes, and the normal appearance of the organism became restored during the course of 12 hr. or less.

4000–9000 lb./sq.in. (272–612 atm.). The higher the pressure, the more rapid was the separation of protoplasm from pellicle. On release of pressure, recovery took several hours. In the experiment illustrated in Pl. 4 the volume appears to have been distinctly smaller after recovery than that before treatment.

10,000 lb./sq.in. (680 atm.). The organism responded within a second of the application of pressure by a separation of the protoplasm from the pellicle. The protoplasm became rounded within a pellicle which appeared bigger than before

(Pl. 5, C2-4, D2 and D4). In a few cases the protoplasm appeared shrunk (Pl. 5, series D; see also Text-fig. 5). The tentacles appeared slightly irregular and a few became obviously bent. On release of pressure after half an hour at 10,000 lb./sq.in. (five experiments) the protoplasm spread within about a minute and made contact with most of the pellicle, following the creases and wrinkles of the latter (Pl. 5, C5 and D3). The protoplasm appeared to occupy the pellicle more fully within the next few minutes, as though swelling. The tentacles became still more bent and irregular, faded away, and apparently disintegrated. Sometimes the meganucleus, normally scarcely visible, became conspicuous on release of pressure (Pl. 5, C5), but later faded. During the course of the following few hours, the perimeter of the organism, as seen in optical section, grew progressively shorter. During this process the pellicle lost its deep folds, became minutely wrinkled (Pl. 5, C7) and finally within a day or two became smooth. However, in several cases, in a region where the protoplasm had failed to make contact with the pellicle, a small wrinkle of pellicle persisted for many hours (Pl. 4, B9). New tentacles appeared as the body surface became smooth again (Pl. 5, C9). *Discophrya* which had been exposed to 10,000 lb./sq.in. for half an hour gave rise to flourishing cultures.

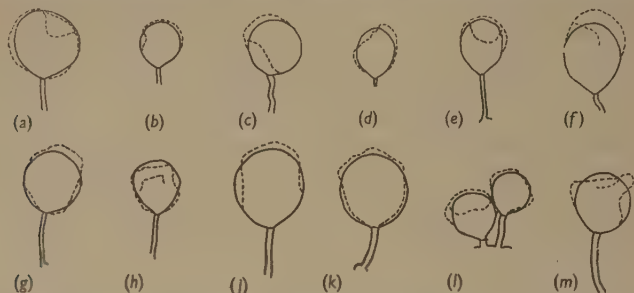
12,000-15,000 lb./sq.in. (816-1020 atm.). The effects on the general appearance were the same as those at 10,000 lb./sq.in. There was usually an immediate rounding up of the protoplasm within the pellicle. The meganucleus usually became visible on release of pressure. The organisms eventually recovered their normal appearance provided that exposure to high pressure had not lasted too long. A few organisms survived a pressure of 15,000 lb./sq.in. for 7 min. The contractile vacuole resumed activity, and the body partly recovered its shape in about 3 days. However, the number of tentacles was small, and the general appearance was abnormal. Other individuals swelled up soon after release of pressure and were obviously dead.

Interpretation of creasing

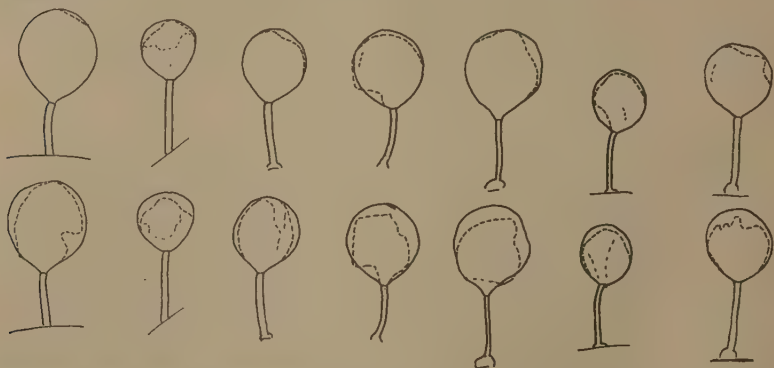
Creasing of the body surface might be due either to a decrease in body volume or to an increase in the area of the body surface. It is not possible to determine changes in volume from the photographs, because the rotational symmetry of the organism is lost with creasing. The general impression obtained from watching the process of creasing is that the organism shrinks in volume. This is strongly suggested by Pl. 4, as well as by some of the superimposed tracings of organisms before treatment and after rounding up of the protoplasm at high pressure (Text-fig. 5). However, it cannot be definitely asserted that creasing at relatively low pressure is accompanied by a loss of volume, because an increase in area equivalent to only a small increase in radius could cause a considerable localized creasing without change in body volume.

Comparisons were made of the outline of *Discophrya* before and after creasing, and the length of the perimeter of the pellicle was measured in each case. Examples in which the protoplasm had not yet substantially separated from the pellicle are given in Text-fig. 3. The perimeter always increased with creasing, and the increase presumably affected the protoplasmic surface as well as the pellicle. For

seven organisms subjected to 10,000 lb./sq.in. for half an hour or longer, so that the protoplasm became partially rounded up within the pellicle, the average increase in perimeter (measured along the pellicle) was 11% with extremes of 4 and 16%.



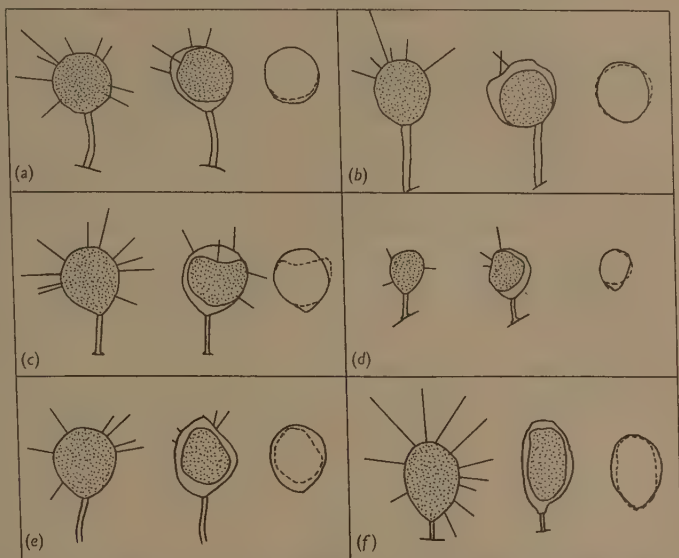
Text-fig. 3. The creasing of *D. piriformis* at high hydrostatic pressure. Tracings from photographs obtained in twelve experiments on different individuals. In each case the continuous lines represent the outline before treatment, and the broken line the outline at high pressure as stated below. The change in length of the perimeter is also given for each case as a percentage of the original length. The protoplasmic surface, in all these cases, was still adhering closely to the pellicle. *a*, 1 min. at 4250 lb./sq.in.; +5% (or 12% along fold). *b*, 15 sec. at 4250 lb./sq.in.; +6%. *c*, 18 sec. at 6100 lb./sq.in.; +4%. *d*, 4 min. at 5000 lb./sq.in.; +10%. *e*, 13 min. at 5000 lb./sq.in.; +9%. *f*, 13 min. at 5000 lb./sq.in.; +17%. *g*, 3 hr. at 3000 lb./sq.in.; +7%. *h*, 3 min. at 6000 lb./sq.in.; +3%. *j*, 2½ min. at 4000 lb./sq.in.; +2%. *k*, 5 min. at 4000 lb./sq.in.; +4%. *l*, 8 min. at 3500 lb./sq.in.; +14% and +17%. *m*, 12 min. at 15,000 lb./sq.in.; +8%.



Text-fig. 4. Shrinkage of *D. piriformis* by exosmosis. Tracings from photographs of six individuals caused to shrink slightly (upper row) and then more drastically (lower row) by treatment for about 20 min. in 0.1 M sucrose, followed by about 30 min. in 0.2 M sucrose. In the upper row the animals are shown before treatment (continuous line) and after treatment with 0.1 M sucrose (broken line); in the lower row the same animals are shown before any treatment (continuous line) and after treatment with 0.2 M sucrose.

In contrast to this, organisms caused to wrinkle by exosmosis in a 0.1 M solution of sucrose always showed a decrease in length of the perimeter, the average for seven cases being 3.6% with extremes of 0.4 and 6% (Text-fig. 4). In a number of

cases the increase in perimeter resulting from high pressure was very obvious, the most extreme being that of the newly settled organism shown in Pl 3, in which the increase in length of the perimeter amounted to 28%. The protoplasmic surface remained adhering to the pellicle in this experiment, and must have increased in area to the same extent. Other examples are illustrated in Pl. 4, series B (increase in perimeter 15%) and Pl. 5, series C (increase 12%).



Text-fig. 5. The rounding up of the protoplasm of *D. piriformis* at high hydrostatic pressure.

Tracings from photographs obtained in six experiments on different individuals. In each case the left-hand drawing shows the outline of the organism before treatment; the centre drawing shows the outline of the organism and of the protoplasm within the pellicle at high pressure, as stated below; and the right-hand drawing shows the outline of the protoplasm (without pellicle) before treatment (continuous line) and at high pressure (broken line). The treatments were as follows: *a*, 30 min. at 10,000 lb./sq.in. *b*, 7 hr. at 6000 lb./sq.in. *c*, 30 min. at 10,000 lb./sq.in. *d*, 32 min. at 10,000 lb./sq.in. *e*, 73 min. at 3000 lb./sq.in. followed by 69 min. at 9000 lb./sq.in. *f*, 73 min. at 3000 lb./sq.in. followed by 72 min. at 9000 lb./sq.in.

Further experiments on creasing

In a few experiments organisms were subjected to pressures normally sufficient to produce creasing, but the pressure was released before creasing occurred. In two cases a pressure of 5000 lb./sq.in. was applied for 5 sec., and in two others 3000 lb./sq.in. for 10 sec. No creasing developed either during the application of pressure or afterwards.

In another experiment a *Discophrya* was subjected for brief periods (3 sec.) to pressures of 5000–6000 lb./sq.in., alternating with longer periods (6–12 min.) without applied pressure. Some wrinkling developed during the exposure to pressure, but not as much as would be expected if the pressure were maintained. During the

interval without applied pressure the wrinkling gradually faded, and eventually disappeared entirely. Pressure was applied and released in this way four times with the same result.

DISCUSSION

Pressure causes an expansion of the pellicle of *Discophrya piriformis*. This process is not immediate, but takes a minute or more, at any rate at the lower pressures. It is not known what causes this expansion, but it seems likely that it depends on some effect of pressure on the underlying protoplasm. The expanded pellicle does not return immediately to its original condition after release of the pressure, but undergoes a slow process of reorganization over a period of hours; contact with the surface of the protoplasm seems to be necessary for this purpose, and possibly surface enzymes play a part. It seems possible that expansion of the pellicle as a result of pressure is of the same nature as expansion during feeding. It is of the same order of magnitude in both cases. Perhaps pressure merely sets in motion a process normal to the life of the organism.

The protoplasmic surface appears also to expand in many cases in which the pressure is not too high. It is not known whether it is drawn out by the expanding pellicle, permitted by the expanding pellicle to exercise an innate tendency to spread, or caused to expand by a direct influence of pressure upon it. The second alternative is perhaps the simplest, and receives some slight support from the observation that protoplasm which has been caused to recede from the pellicle or round up at high pressure usually spreads and makes contact with the pellicle within a few minutes after release of the pressure. A capacity of the protoplasmic surface to expand might provide a basis for suction.

Prolonged exposure to high pressure usually causes a recession of the protoplasm from the pellicle, and at rather high pressures the protoplasm may become partly rounded up. This may be comparable with the rounding up of amoebae (Marsland & Brown, 1936), and the retrogression of cleavage furrows in sea-urchin eggs (Marsland, 1938) at high pressure, and in terms of current theories of the arrangement of cell proteins (Goldacre & Lorch, 1950; Mitchison, 1952), may be ascribed to a folding of protein molecules to a contracted and possibly even globular state. The paradox of expansion at moderate pressure and contraction at higher pressures may be explained in various ways, as for instance by supposing that moderate pressures expand the pellicle and allow the protoplasmic surface to expand, but that higher pressures affect the protoplasmic surface directly and cause it to contract.

The whole discussion of the effects of high pressure on *Discophrya* is complicated by the fact that certain of the records suggests a decrease in body volume, but there is considerable variation in this matter. The change in volume due to the compressibility of water would not be noticeable and cannot explain these cases. There must be a loss of material from the organism. Water might for some unknown reason pass out through the tentacles, or perhaps pressure might produce a redistribution of water between the organism and environment owing to the volume change of concentration or dilution of cell solutes. It is not possible to say whether

a decrease in volume contributes significantly to creasing at the lower pressures, but the fact that creasing is fully effective at pressures too low to cause a serious decrease in the rate of output of the contractile vacuole indirectly but strongly suggests that there is no loss of water from the hyaloplasm.

It would be imprudent to speculate any more on the effects of high pressure on *Discophrya* until a comparison can be made with other materials.

SUMMARY

1. The suctorian *Discophrya piriformis* Guilcher has been subjected to pressures ranging from 1000 to 15,000 lb./sq.in. (68–1020 atm.).

2. Pressures of 2000 lb./sq.in. and over cause a creasing of the body surface. Except at the higher pressures this occurs some seconds after the application of pressure.

3. Creasing is accompanied by an expansion of the pellicle. At the lower pressures, there is also an expansion of the protoplasmic surface. A comparison may be made with the expansion of the body surface which occurs during feeding.

4. In many cases, and particularly at the higher pressures, the protoplasm later separates from the expanded pellicle. With prolonged treatment it sometimes rounds up, and there is evidence of a loss of volume of the protoplasm.

5. On release of pressure the protoplasm spreads back to the pellicle, usually within a few minutes. The wrinkled and expanded pellicle is then slowly re-organized to its normal shape and size, over a period of many hours.

I am greatly indebted to the Alkali Division of Imperial Chemical Industries Ltd., through the courtesy of which I received much help from Mr G. A. Shephard, Mr H. J. Welbergen and Mr T. A. Wych. Mr Shephard designed the pressure vessel, Mr Welbergen made the glass windows for it after a series of trials, and Mr Wych made the metal parts for the windows and tested the windows at pressure. Imperial Chemical Industries Ltd. (Alkali Division) provided the high tensile steel and other components for the pressure vessel.

I am grateful to Dr J. M. Burch for polishing many windows, to Mr H. Banwell for making the pressure vessel, to Mr M. Gillet for modifying a microscope for use with it, and to Mr E. Livingstone for culturing *Discophrya*.

It is a pleasure to thank Dr C. R. Burch for most valuable help and advice in optical matters and Prof. J. E. Harris for many useful suggestions and for helpful criticism of the manuscript.

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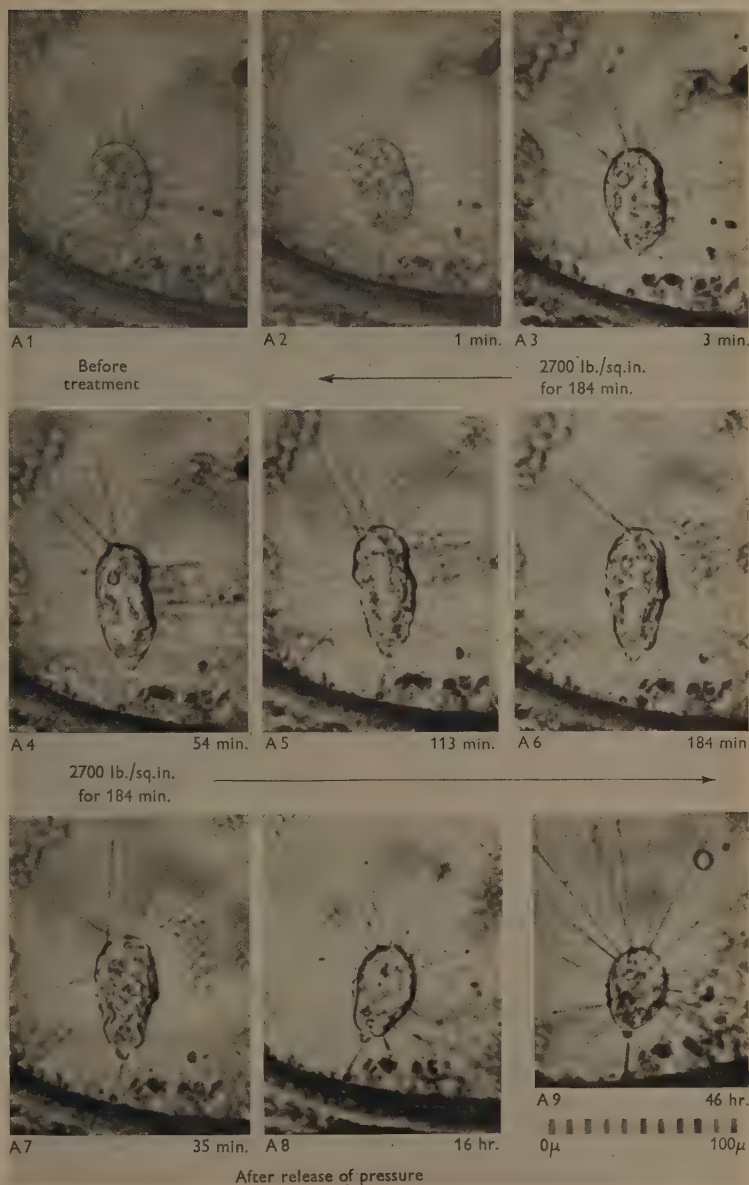
EXPLANATION OF PLATES

PLATE 3

Series A. An experiment in which *D. piriformis* was subjected to 2700 lb./sq.in. for 184 min. This series illustrates to an exceptional degree the expansion of the body surface and pellicle at high pressure, as well as the slow recovery of the normal appearance after release of pressure. During reorganization of the pellicle small folds remained, and are visible on the left side in A8 and A9. The contractile vacuole is visible in A3 and A4, and in some of the other photographs.

PLATE 4

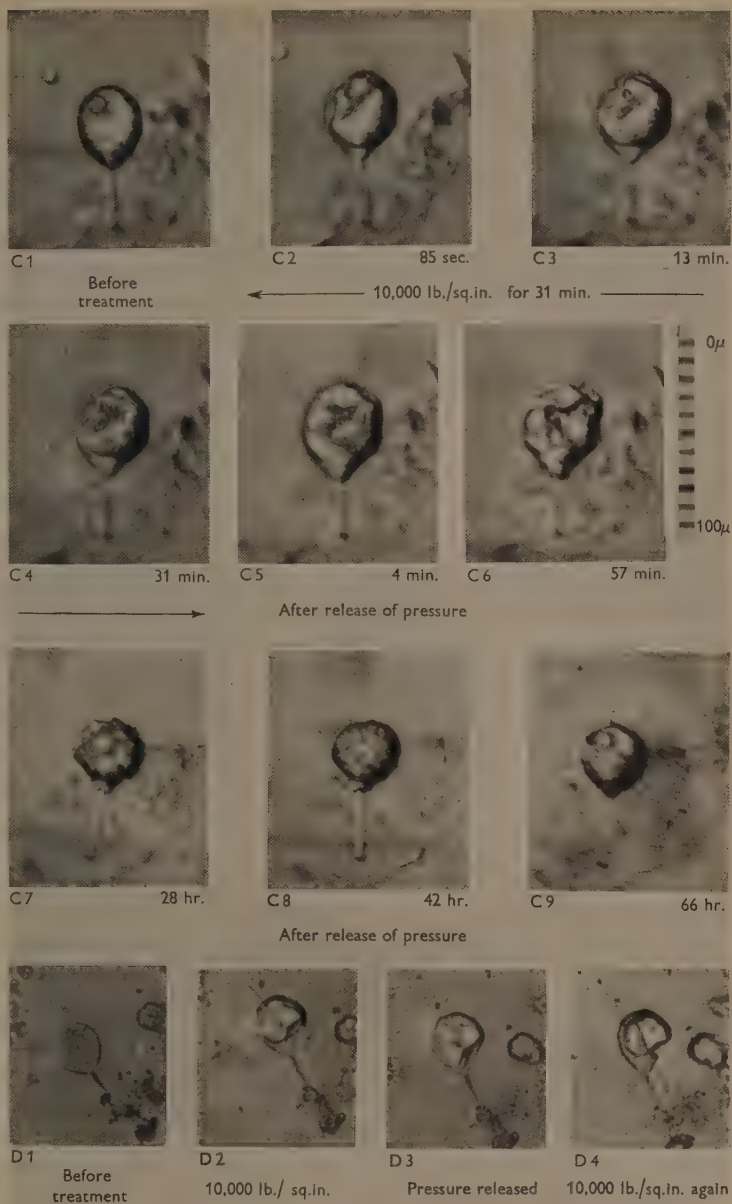
Series B. An experiment in which *D. piriformis* was subjected to 3000 lb./sq.in. for 27 min., followed by 9000 lb./sq.in. for 7 min. There was no creasing 10 sec. (B2) after application of 3000 lb./sq.in., but it was far advanced after 3 min. at this pressure (B3). The protoplasm receded from the expanded pellicle and appears to have decreased in volume (B4). A short exposure to 9000 lb./sq.in. produced little further change (B5 and B6). On release of pressure the protoplasm made contact with the overlying pellicle (B7 and B8), and the normal body shape was restored, with shrinkage of the pellicle. However, a small fold remained where the protoplasm and pellicle were not in contact (B9). The body was slightly smaller than before the experiment (B9 and B1).



KITCHING—THE EFFECTS OF HIGH HYDROSTATIC PRESSURES
ON A SUCTORIAN



KITCHING—THE EFFECTS OF HIGH HYDROSTATIC PRESSURES
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ON A SUCTORIAN

PLATE 5

Series C. An experiment in which a *D. piriformis* was subjected to 10,000 lb./sq.in. for 31 min. The protoplasm rounded up within an expanded pellicle (C3), and some of the tentacles became bent and irregular (C4). On release of pressure, the surface of the protoplasm re-expanded under the pellicle (C5) and the latter became thrown into folds which were everywhere in contact with protoplasm (C6). The protoplasmic surface and pellicle were slowly reorganized to their normal shape and smoothness (C7-9). The old tentacles disappeared (C6) and new ones were formed (C9). The contractile vacuole is visible in C1 and in some of the other photographs. The meganucleus is just visible in C5, occupying one-third of the width of the organism.

Series D. An experiment in which *D. piriformis* was subjected to 10,000 lb./sq.in. for 10 min., released to atmospheric pressure for 13 min. and then recompressed to 10,000 lb./sq.in. D1, before treatment; D2, after 10 min. at 10,000 lb./sq.in.; D3, 1 min. after release of pressure; D4, 5 min. after recompression to 10,000 lb./sq.in. The pellicle is slightly folded and appears slightly larger, and the protoplasm is rounded within it, in D2. The protoplasm has spread within the pellicle and has made contact everywhere with it in D3. The protoplasm has rounded up again in D4.

THE PHYSIOLOGY OF CONTRACTILE VACUOLES

IX. EFFECTS OF SUDDEN CHANGES IN TEMPERATURE ON THE CONTRACTILE VACUOLE OF A SUCTORIAN; WITH A DISCUSSION OF THE MECHANISM OF CONTRACTION

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(Received 1 June 1953)

(With Three Text figures)

INTRODUCTION

When the suctorian **Discophrya piriformis* Guilcher (1947) is transferred to a medium of different osmotic pressure, there is a slight delay in the adjustment of the rate of output of the contractile vacuole to a new osmoregulatory level. It has been suggested that the rate of osmotic uptake of water from the medium into the organism must change simultaneously with the change of medium, and therefore that the lag in vacuolar response must cause a slight change in body volume, which may itself mediate the change in rate of vacuolar output. The calculated change in body volume is too small to be demonstrated directly by measurement (Kitching, 1951).

It has also been shown that the rate of vacuolar output of the peritrich ciliate *Carchesium aselli* increases with a rise in temperature within the range 0–34° C. (Kitching, 1948a). Moreover, it has been concluded from indirect evidence that an increase in temperature causes an increase in the permeability of the body surface to water, resulting in an increased rate of osmotic uptake of water into the organism, which in turn would provoke an increase in the rate of vacuolar output (Kitching, 1948b). It is possible also that there might be a direct effect of temperature on the rate of vacuolar secretion or on the response of this to small changes in the body volume. It therefore seemed to be of interest to study the effect of sudden changes of temperature on the activity of contractile vacuoles. *Discophrya piriformis* was chosen as material because its frequency of systole is convenient for studies of this kind. Much work has already been done on the effect of temperature on vacuolar frequency, and references are given by Kitching (1938). These observations are difficult to interpret because the diameter of the vacuole at systole was not measured.

METHODS

Discophrya piriformis was grown as usual on silk threads and fed on *Paramecium* or *Colpidium*. Fairly small individuals were used, having one contractile vacuole.

During experiments *Discophrya* was kept in a continuous flow of Bristol tap water, supplied at the temperature required from a supply tube fitted with a water

* Called *Podophrya* sp. in earlier papers of this series.

jacket, as already described (Kitching, 1934). The apparatus is shown in Fig. 1. The silk threads carrying the *Discophrya* were looped around the fine thermocouple wire, and the *Discophrya* used for observation lay with its long axis in the optical plane and within 2 mm. of the thermocouple junction. Two supply tubes, to provide water at different temperatures, were clamped in position as shown, and when the temperature was to be changed one tube was shut off and the other turned on. The heat capacity of the apparatus was very low, as the specimen rested on a long cover-glass instead of on a slide. With brisk flow a rise of 10° C. was 90% complete within 10 sec.

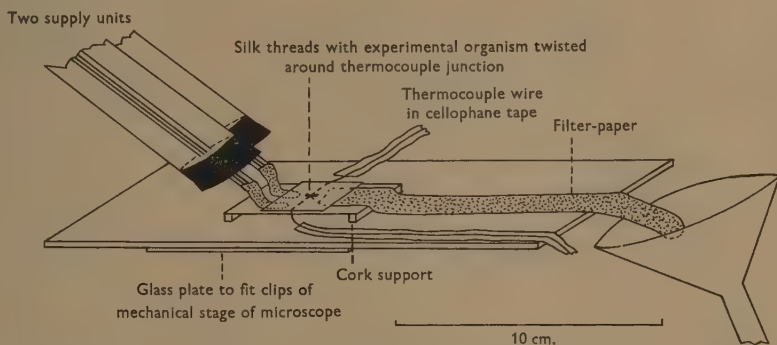


Fig. 1. Irrigation apparatus for producing sudden changes of temperature. Details of the supply units are given elsewhere (Kitching, 1934).

The *Discophrya* was acclimatized under experimental conditions at the initial experimental temperature for at least 45 min. before observations were begun.

RESULTS

Some 10–20 min. after a change of temperature, the rate of secretion of fluid into the contractile vacuole and the frequency of systole were found to have increased with temperature within the range $5\text{--}30^{\circ}$ C. There was little change in the ultimate diameter, although this was rather variable at the lower temperatures. The experiments described below are concerned with the process of transition to the new vacuolar rhythm. Twenty-nine experiments were carried out, comprising seventy-five sudden changes of temperature.

After a sharp rise in temperature from below 15° C. (Fig. 2) the current vacuolar cycle was terminated by systole rather more quickly than would otherwise have been the case. The average rate of output for this cycle was found to be high, although the ultimate diameter was smaller than before the rise in temperature. Occasionally one or two additional cycles followed quickly, of short duration, small ultimate diameter and rapidly decreasing rate of output. In any case within a few minutes or less the activity of the contractile vacuole was severely reduced. Often no vacuole was visible at all for several minutes, and in other cases the vacuole was

very small, and grew only very slowly. In a number of experiments the body was seen to become smoother in outline during this period, small dimples filling up as though the body were swelling slightly. Vacuolar activity was then gradually

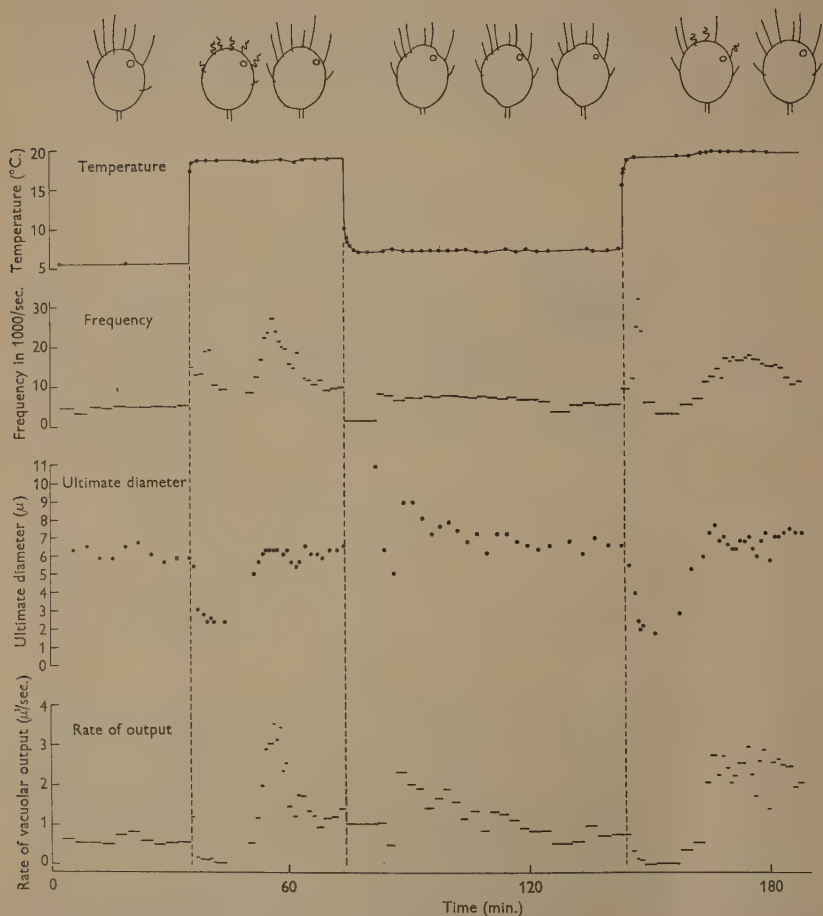


Fig. 2. Effects of sudden changes of temperature on the vacuolar cycle of *Discophrya piriformis* Guilcher; initial temperature low.

restored, with the rate of output, ultimate diameter, and frequency all increasing until a new steady rate was attained, characteristic of the new temperature.

The description given above holds good for all cases in which the initial temperature was below 15°C.; the rise was at least 5°C. In experiments in which the

initial temperature was above 20° C., there was no temporary depression of activity (Fig. 3).

After a sharp fall in temperature (Fig. 2) the current vacuolar cycle was greatly prolonged. The average rate of secretion was usually about the same as before, but

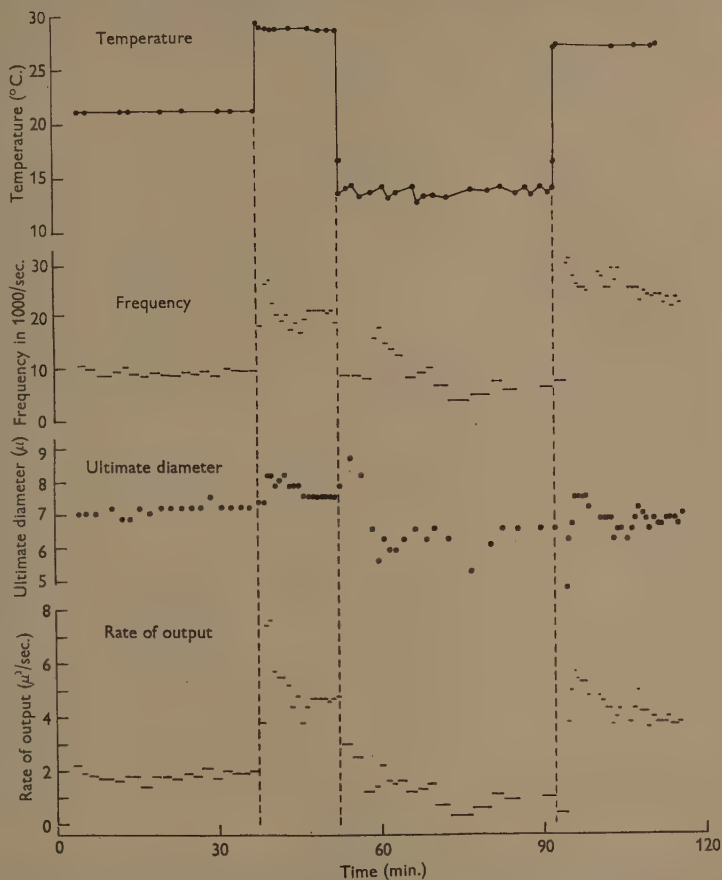


Fig. 3. Effects of sudden changes of temperature on the vacuolar cycle of *Discophrya piriformis* Guilcher; initial temperature rather higher than in Fig. 2.

the ultimate diameter was much greater; this fact increases the error in determining the rate of output. Systole, when it occurred, was always very slow, sometimes taking several minutes; occasionally it was incomplete. During the next few vacuolar cycles the rate of secretion and ultimate diameter decreased, and the frequency increased, until the steady condition characteristic of the new low

temperature was attained. Sometimes during the first few cycles after a fall in temperature several vacuoles were seen to grow in the position of the contractile vacuole and only fused to form the contractile vacuole proper after they had reached a considerable size. This was particularly noticeable at the lowest temperatures (5–10° C.). In three experiments dimples appeared in the body surface during the first few vacuolar cycles after the fall in temperature, as though the body were shrinking. In one case it was possible to observe the increase in depth of these dimples with each successive systole.

DISCUSSION

Control of vacuolar secretion

The body volume depends on a balance between entry and evacuation of water. If a change of temperature affects these two equally, the body will not change in volume; but if unequally, it will. However, a change in volume will automatically evoke a change in vacuolar output in such a direction as to regulate the body volume towards its original level. At 15° C. a decrease of 1½ % of body volume is believed to be sufficient to stop vacuolar secretion completely (Kitching, 1951), so that the control of body volume is delicately set. Temperature might affect the sensitivity of this control, but even so it seems likely that a very small change of body volume would be sufficient to correct any differential action of a change of temperature on the uptake and output of water by the organism.

Actually, immediately after an increase in temperature from below 15° C., there is a severe depression of vacuolar secretion. A small amount of water, perhaps already mobilized in contributory vacuoles, is rapidly discharged in some cases, and then secretion is depressed or even almost halted for several minutes. During this period the body swells noticeably; water is probably already entering the body at the increased rate characteristic of the higher temperature. Finally, vacuolar secretion is resumed, perhaps partly in response to the increase in body volume, and a new steady state is established with a greater turn-over of water than before.

When the temperature is suddenly decreased, the rate of vacuolar output may continue for a short time at a relatively high level, but eventually decreases. In several cases during the period of adjustment a definite though slight decrease of body volume was seen. This decrease may perhaps partly determine the decrease in rate of vacuolar output.

Control of systole

The vacuolar cycle has three characteristics: the rate of secretion of water into the vacuole (whatever the mechanism may be), the ultimate or maximal diameter, and the frequency of discharge. All three may vary, but only two may vary independently. For instance, both in peritrich ciliates and in *Discophrya piriformis* the frequency, ultimate diameter and rate of secretion all increase with a greater osmotic inflow of water from the external medium into the organism. The interpretation of the changes in the vacuolar cycle described in this paper can only be attempted after some speculation as to how these three characteristics of the vacuolar cycle are determined.

The rate of secretion is known to be controlled in accordance with osmotic conditions (Kitching, 1951), and the other two characteristics must together conform to it. If changes in the rate of secretion found their expression entirely in a change either of frequency or of ultimate diameter, the other remaining constant, a simple hypothesis could be devised; but this is not the case. The possibilities therefore remain of supposing either that under constant conditions there is a particular ultimate diameter at which, for mechanical reasons, the vacuole contracts, but that this critical diameter is modified by a change of conditions, or that there is a rhythmic process causing systole at regular intervals, but that the rhythm is subject to modification.

In *Amoeba* it appears that the contractile vacuole discharges when it reaches and effects the necessary contact with the plasmalemma. In *Euplotes* also the main vacuole discharges when it makes contact with the pore (Taylor, 1923). It is therefore possible to suppose that in these cases systole is initiated by the piercing or unplugging of the pore and is effected by surface tension or body turgor or the elasticity or contraction of the vacuolar wall, as soon as these are given an opportunity to act. However, in some ciliates, such as the Ophryoscolecidae (MacLennan, 1933), each contractile vacuole remains attached to its pore for a large part of the vacuolar cycle, and yet only contracts when fully grown. Moreover, in one animal there are several vacuoles, and only the full one contracts. Body turgor would affect them all alike, and surface tension would exert the greatest pressure on the smallest vacuole, so that here again there must be some other mechanism by which systole is initiated. MacLennan (1933) has reported that just before systole the protoplasm around the contractile vacuole solates, as judged by Brownian movement, and he has suggested that associated with this there is a solation of the pore plug, permitting body turgor or surface tension to discharge the vacuole. Systole is known to occur in *Paramecium* when the body is partly shrunk (Kitching, 1952) and in *Discophrya* when the body is wrinkled (Kitching, 1951; and 1954, fig. 1), so that in these cases at any rate body turgor is not necessary. Surface tension might well provide the small pressure required (Kitching, 1952), provided that the vacuolar wall is liquid. In *Amoeba proteus* the vacuolar wall is about $\frac{1}{2}\mu$ thick (Mast, 1938), and when an amoeba (*A. lacerata*) is squashed the vacuole may persist for some time suspended in the surrounding water (Hopkins, 1946). The vacuolar margin is weakly birefringent (Schmidt, 1939). The vacuolar wall therefore clearly has structure, and this structure is likely to be of protein. In the absence of evidence as to the nature of the structure, further comments are purely speculative. However, the vacuolar contents may be regarded as a foreign phase, physiologically external and likely to be surrounded by a plasma membrane, perhaps with protein molecules on the cytoplasmic side oriented in one of the ways discussed by Mitchison (1952, fig. 2). These would provide some elasticity, and if extended could contract as suggested by Schmidt (1939). It seems possible that the solation observed by MacLennan is part of a general condition affecting the whole vacuolar apparatus in such a way as to contract protein molecules ultimately to the globular state, in accordance with the cycle suggested for amoebae by

Goldacre & Lorch (1950). This might initially produce an increase in tension in the wall of the vacuole, thus causing the rounding up prior to systole illustrated by MacLennan (1933, fig. C) and also found in various peritrich ciliates. As the protein molecules became globular and passed into the surrounding hyaloplasm, surface tension might take over and complete the process of systole.

It is necessary, and teleologically desirable, to admit a connexion between the frequency of contraction and the rate of secretion. Both are increased by a greater osmotic inflow of water from the medium into the organism. Various possibilities are: (1) that there is a common source of energy or a common component in the two mechanisms, (2) that the small changes in volume which probably control the rate of secretion also affect the consistency of the pore plug or the structure and properties of the vacuolar wall, (3) that extension of the vacuolar wall increases the tendency to discharge and so accelerates the rhythm.

The observations on *Discophrya* described in this paper will now be considered. An increase of temperature ultimately causes an increase in vacuolar frequency, just as in *Carchesium aselli* (Kitching, 1948a) and in many other Protozoa. This increase may perhaps be ascribed to an increase in velocity of chemical reactions promoting contraction and solation. However, the immediate effect of a rise of temperature from below 15° C. (Fig. 1) is to depress the secretion of fluid into the contractile vacuole. The first one or two systoles after the rise in temperature occur very quickly, but with a decrease in ultimate diameter; perhaps the timing mechanism has been accelerated, but available water is running short. Then there is a slowing down to a very low frequency. The rise in frequency has been counteracted by the depression in rate of secretion, frequency and secretion being in some way linked, as already explained. Finally, when secretion is resumed, the frequency is restored to the high level which is normal for that temperature.

After a sudden fall of temperature the contractile vacuole continues to grow in volume at a fairly rapid rate, but systole is long delayed, so that the ultimate diameter is much greater than usual. The frequency then increases, and the ultimate diameter decreases, to the steady level characteristic of the lower temperature. Following the analysis by Burton (1939) of overshoot in a chain of chemical reactions, it is possible to suggest that the fall in temperature drastically slows down a chemical process upon which contraction depends, but that as a result of this the products of earlier reactions in the chain pile up and by the law of mass action once more accelerate the rhythm. This concept has been applied to rhythmic processes and neatly demonstrated by Wells & Ledingham (1940). In the case of *Discophrya*, any overshoot of the rise of vacuolar frequency with increase of temperature would be masked by the depression in secretory activity; however, there is some suggestion of it in Fig. 2.

SUMMARY

1. A study has been made of the effects of sudden changes of temperature on the contractile vacuole of the suctorian *Discophrya piriformis* Guilcher.
2. A sudden increase of temperature from below 15° C. by 5° or more causes a temporary fall in the rate of output, followed by a rise to a new level higher than the original. During the depression in activity the body swells slightly.
3. The vacuolar frequency increases immediately but briefly when the temperature is raised, falls steeply when the depression sets in, and when secretion is re-established rises again to a level above the original.
4. A sudden fall in temperature causes an immediate decrease in vacuolar frequency, followed by a partial restoration. The rate of output falls rather more slowly and remains low. In several cases a small decrease in body volume was observed.
5. It is suggested that the contractile vacuole is really contractile.
6. The observations on vacuolar frequency described in this paper are interpreted in terms of an inherent vacuolar rhythm which is modified by temperature and which is partially linked with rate of secretion.

I am grateful to Prof. J. E. Harris for his helpful criticism of the manuscript. I wish also to thank Mr M. Gillett for taking galvanometer readings during the course of these experiments.

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THE PHYSIOLOGY OF CONTRACTILE VACUOLES

X. EFFECTS OF HIGH HYDROSTATIC PRESSURE ON THE
CONTRACTILE VACUOLE OF A SUCTORIAN

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(Received 1 June 1953)

INTRODUCTION

The effects of high pressure on living material are little understood and probably complicated. In a number of cases the tendency of cytoplasmic inclusions to become stratified by centrifuging is increased, and this is attributed to a decrease in protoplasmic viscosity (Marsland, 1942). High pressure is therefore considered to solate protoplasmic gels. In addition, high pressure affects contractile structures; at ordinary temperatures it increases the tension of muscle during contraction (Brown, 1934, 1936) and it temporarily increases the frequency of beat of certain cilia (Pease & Kitching, 1939). The effect of pressure on the rate of beat of tadpole heart tissue depends upon temperature; increased pressure causes a temporary increase in frequency at temperatures above 16° C., but a decrease below 14° C. (Landau & Marsland, 1952). It appears that several reactions or processes are differentially affected. It seems likely that all the effects mentioned, including the solation of protoplasmic gels, have a common basis in the folding of protein molecules. This might be caused either directly or perhaps indirectly, through some contraction-causing reaction in which ATP might be concerned.

It has been suggested by Taylor (1923) that sol-gel changes are involved in the cycle of activity of contractile vacuoles, and cyclic changes occur in the viscosity of the neighbouring protoplasm, as judged by Brownian movement, in the Ophryoscolecidae (MacLennan, 1933). MacLennan attributed systole to a solation of the pore plug. It seems likely that the configuration of proteins in the vacuolar wall also plays an important part in systole and is perhaps responsible for the contraction (Kitching, 1954*b*). Moreover, according to Goldacre (1952), the contraction of protein molecules also offers a possible mechanism for secretion. It therefore seemed of interest to study the effects of high hydrostatic pressure upon vacuolar activity.

The suctorian *Discophrya piriformis* Guilcher (1947) was chosen for this work because it is extremely convenient material. It has rotational symmetry, it remains completely still, and its protoplasm is clear except soon after a meal. Pressures of 2000 lb./sq.in. (136 atm.) or more cause a wrinkling of the body surface, which is associated with an expansion of the pellicle and sometimes with a decrease in protoplasmic volume (Kitching, 1954*a*). After release of pressure the normal body shape is slowly restored over a period of many hours.

METHODS

Discophrya piriformis was cultured in Bristol tap water and fed on *Paramecium* spp. and *Colpidium* sp. No food was given for several days before an experiment.

The pressure vessel and associated equipment have already been described (Kitching, 1954*a*). The glass windows are set in steel disks which can be removed. The vessel was filled with medicinal paraffin ('mineral oil'). A small drop of the culture medium containing *Discophrya* was placed on the inner side of one of the windows, and the window was mounted in position in contact with the medicinal paraffin. Pressure was applied from a hydraulic pump in from 1 to 3 sec., and was released in 1 sec. or less.

The technique of observation was as described in previous papers of this series. Normally it was possible to choose a *Discophrya* with only one contractile vacuole, the experiment illustrated in Fig. 2 being an exception. Observations were carried out before treatment, at one or more high pressures, and after release of pressure. In several cases the organism was re-examined the following day, and was apparently healthy. The room temperature was between 15.7 and 20.0° C., and varied very little in any one experiment.

The drawings shown with the graphs (Figs. 1-3) were reconstructed from rough sketches and measurements made hastily during the course of the experiments. They are intended to show the general condition of the animal, and may not be accurate in detail.

RESULTS

Eleven experiments were carried through successfully, and are summarized in Table 1. Some others had to be abandoned because the contractile vacuole was obscured by the creasing of the body surface, which always occurs at pressures of 2000 lb./sq.in. or more. Three experiments are illustrated in Figs. 1-3.

The rate of vacuolar output was depressed at all pressures from 1000 lb./sq.in. (68 atm.) upwards, but the effect was slight in the lowest part of the range (Fig. 1). No vacuolar activity could be detected at 7000 lb./sq.in. (476 atm.) or over. On release of pressures of 1000-5000 lb./sq.in. (68-340 atm.) the rate of output recovered almost immediately to about normal (Figs. 1, 2). After 10,000-14,000 lb./sq.in. (680-952 atm.) there was some delay before the contractile vacuole started working again (Fig. 3), and when that delay was considerable the protoplasm appeared to swell within the wrinkled pellicle and to fill it out. There was no recovery after 16 min. at 15,000 lb./sq.in. (1020 atm.) in experiment 210452*a* (Table 1); in other observations a small proportion of individuals showed some recovery of vacuolar activity after 7 min. at this pressure (Kitching, 1954*a*). This treatment is therefore close to the limit.

The vacuolar frequency was rather variable at pressures below 2000 lb./sq.in. (136 atm.), and no particular significance can be attached to the changes observed. At 2000-3000 lb./sq.in. (136-204 atm.) the frequency increases markedly, and the increase was maintained until the pressure was released (Fig. 1). There appears to have been no correlation with creasing of the pellicle, as in experiment 020352*b*

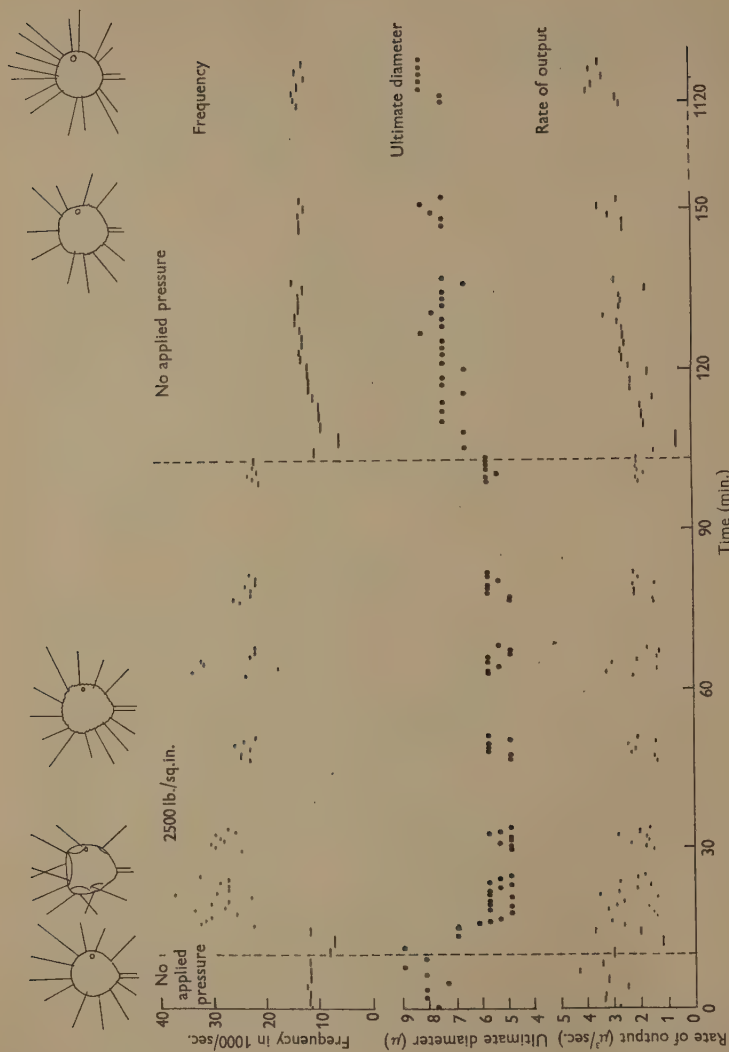


Fig. 1. Effects of a pressure of 2500 lb./sq.in. (170 atm.) on the activity of the contractile vacuole of *Discophrya piriformis*.

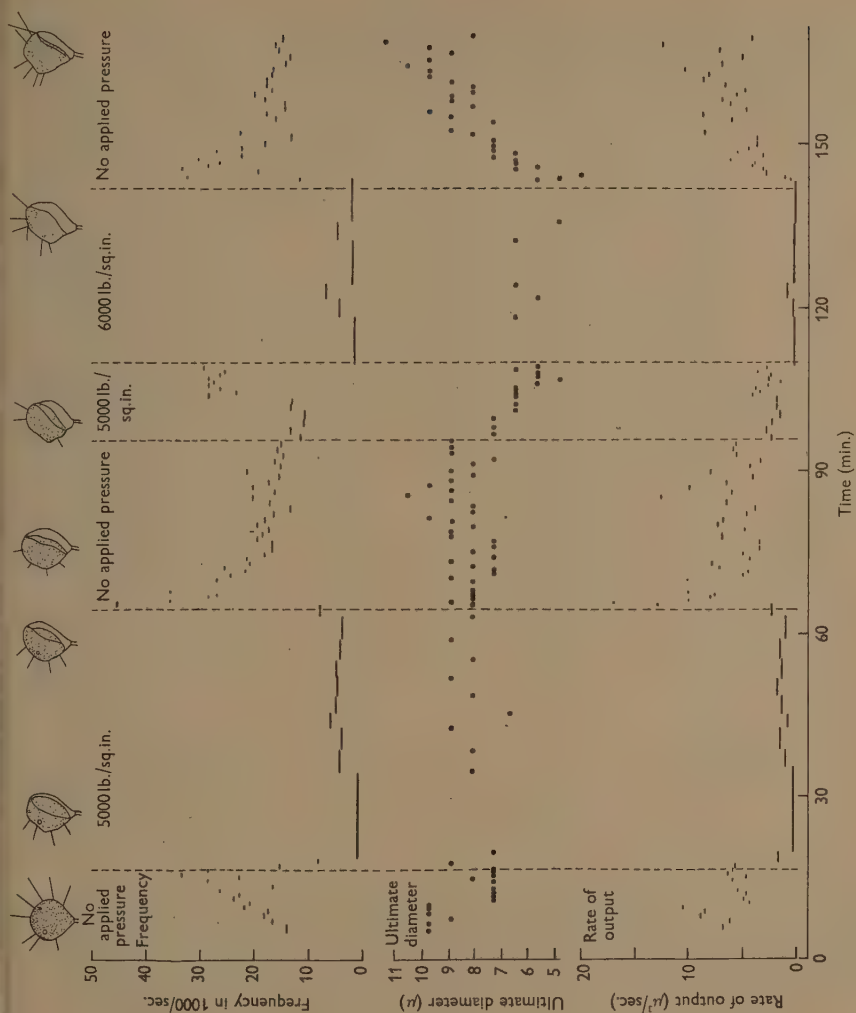


Fig. 2. Effects of pressures of 5000 and 6000 lb./sq.in. (340 and 408 atm.) on the activity of the contractile vacuole of *Discophrya pyriformis*. The larger of the two contractile vacuoles was studied. The other, smaller and less active, behaved qualitatively in the same way.

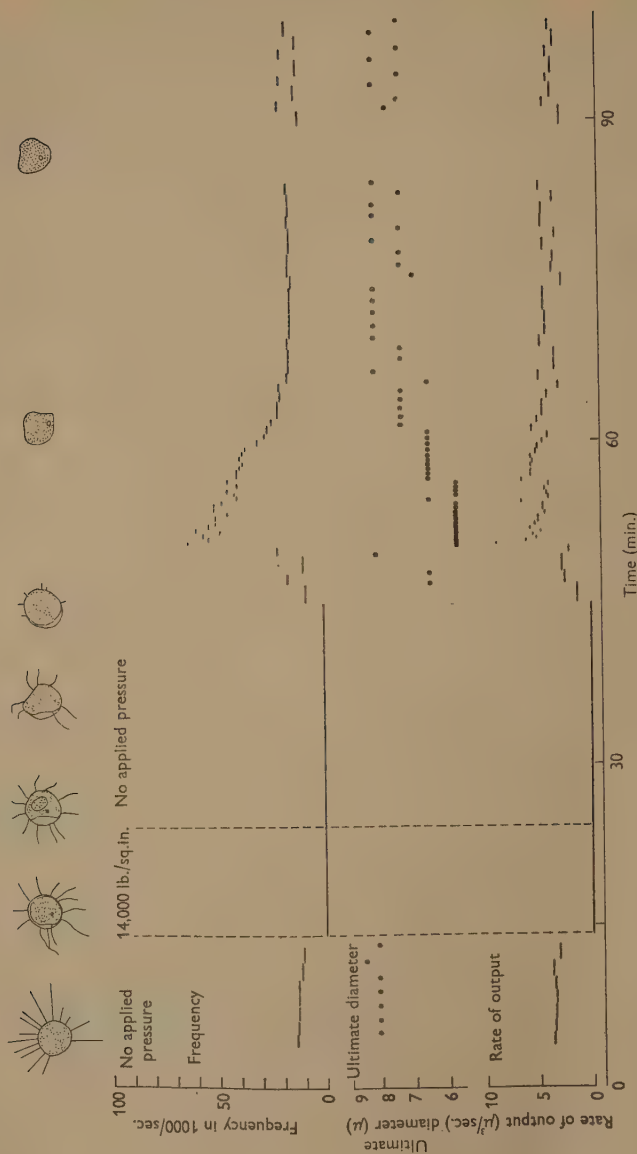


Fig. 3. Effects of a pressure of 14,000 lb./sq.in. (952 atm.) on the activity of the contractile vacuole of *Discophrya piriformis*. In this case the animal is viewed from the end remote from the stalk.

Table 1

Experiment	Pressure applied (lb./sq.in.)	Duration of treatments (min.)	No. of readings contributing to mean*	Mean duration of vacuolar cycle (sec.)	Mean rate of vacuolar output (μ^3 /min.)	Standard error applicable to previous column
260352	0	11	5	128	2.27	0.03
	1000	30	16	112	1.87	0.01
	0	30	16	102	3.23	0.66
230452a	0	23	13	72	2.36	0.08
	1600	30	20	88	2.11	0.25
	0	21	10	127	2.17	0.09
230452b	0	11	7	85	3.26	0.19
	2500	93	52	40	2.05	0.08
	0	49	18	80	2.63†	0.09
020352b	0	20	6	183	1.13	0.04
	2000	31	27	65	0.87	0.05
	0	47	8	139	1.11	0.10
	3000	60	17	68	0.95	0.05
	0	29	6	209	0.85	0.15
240352	0	15	7	116	2.03	0.12
	3400	241	53	53	1.55	0.23
	0	62	28	51	3.24	0.12
210352‡	0	11	13	45	6.4	0.53
	5000	48	8	213	1.38	0.10
	0	31	27	56	6.0	0.36
	5000	14	15	Fell from 90 to 35	2.68	0.65
	6000	32	5	316	0.46	0.45
040452	0	28	23	57	6.45†	0.47
	0	26	8	186	0.88	0.08
	7200	19	—	∞	0.00	—
070452	0	62	8	78	0.49†	0.07
	0	12	5‡	143	4.30	0.04
	8200	21	—	∞	0.00	—
150452	0	66	37	85	3.20	0.26
	0	15	7	134	1.33	0.48
	12000	37	—	∞	0.00	—
210452b	0	94	64	55	2.85	0.15
	0	10	7	81	3.60	0.09
	14000	10	—	∞	0.00	—
210452a	0	75	62	Rose from 20 to 80	§	—
	0	12	6	108	2.82	0.05
	15000	16	—	∞	0.00	—
	0	112	—	Dead	0.00	—

* Two or three readings taken immediately after a change of pressure have been discarded if a steady level was not attained immediately. The duration of treatment given in the previous column is not that used for computation of the mean rate of output, as it includes breaks in the observations.

† Active the next morning also.

‡ The larger of two contractile vacuoles was observed, and the readings given apply to this one only. The other one behaved qualitatively in the same way.

§ Downward drift, see Fig. 4.

|| No recovery; dead next morning.

(Table 1) the increased frequency was maintained throughout the period of exposure to 2000 lb./sq.in. even though the pellicle completely lost its wrinkles during the later part of this treatment. This conclusion is also supported in Fig. 1. Moreover, the frequency decreased to about its original level on release of pressure

in the second part of experiment 020352*b*, even though the pellicle remained creased. At pressures of 5000–6000 lb./sq.in. (340–408 atm.) the frequency decreased considerably, but after release of pressure it rose sharply to a peak before becoming steady. After release from a pressure of 7000 lb./sq.in. (476 atm.) or more, when the vacuole resumed activity the frequency rose to a level considerably above the original.

DISCUSSION

It has been suggested that systole is associated with a folding up of protein molecules, ultimately to globular form, this process providing not only for the opening of the pore but also for the contraction of the vacuolar wall and its ultimate solation and disappearance (Kitching, 1954*b*). The nature of the hypothetical protein structure in the vacuolar wall is unknown. The increase of vacuolar frequency at 2000–3000 lb./sq.in. accords well with this suggestion, as it might reduce the stability of the pore plug, promote contraction and solation of the vacuolar wall, or influence the chemical processes controlling the rhythm of contraction. At higher pressure the rate of secretion is considerably depressed, and with it the vacuolar frequency. The linkage between the two processes has already been noted and discussed (Kitching, 1954*b*).

Marsland (1950) found that cortical gelation in the eggs of *Arbacia* decreases with pressure but increases with temperature, these two influences acting in opposition. On the other hand, they both act together in accelerating the vacuolar rhythm of *Discophrya*. In the latter case we are probably concerned with a rhythmic change in protein configuration instead of a steady state of solation and gelation. The frequency (and the amplitude) of the isolated frog's heart is increased at moderately high pressure (60 atm.) (Edwards & Cattell, 1928).

It is too soon to discuss the various other observations recorded in this paper.

SUMMARY

1. The contractile vacuole of the suctorian *Discophrya piriformis* Guilcher has been observed at hydrostatic pressures ranging up to 15,000 lb./sq.in. (1020 atm.).
2. The rate of output of water was reduced at high pressures, and vacuolar activity was entirely suppressed at about 7000 lb./sq.in. (476 atm.).
3. The vacuolar frequency was increased at 2000–3000 lb./sq.in. (136–204 atm.), but returned to normal on release of pressure. The frequency was depressed at 5000 lb./sq.in. (340 atm.) or more, but on release of pressure rose to a level higher than before treatment.
4. The increase in vacuolar frequency at moderate pressures is perhaps comparable to the increase in tension of muscle at these pressures. High pressure probably promotes instability of the pore plug, and contraction and solation of the vacuolar wall.

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SLOW CONTRACTION AND ITS RELATION TO SPONTANEOUS ACTIVITY IN THE SEA-ANEMONE *METRIDIUM SENILE* (L.)

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It has been known for some time that the most rapid reflex contractions of sea-anemones show a characteristic relation to the stimulus. Thus in *Calliactis parasitica* a short series of adequate electric shocks, given at a frequency of about 1 per sec., is regularly followed by a facilitated contraction of the marginal sphincter (Pantin, 1935*a, b*). That is, each successive shock sets up a nervous impulse which facilitates the response of the muscle to the next; so that the contraction increases by steps corresponding to the shocks. The facilitating effect of each impulse decays so that as the frequency of stimulation is lowered the response becomes smaller and finally vanishes.

But in *Calliactis* stimulation at frequencies which are too low to cause the sphincter response nevertheless succeeds in calling certain other very slow systems of muscles into action. The mesenteric retractors, the longitudinal parietal muscles, and the circular muscle of the body wall seem to be excited by successively lower frequencies (Pantin, 1935*a*). Hall & Pantin (1937) found a rather similar facilitation sequence in *Metridium senile*, though in this case it was the mesenteric retractors which responded most rapidly and to the highest frequency of stimulation.

It was at first supposed that the responses of these slow muscle systems were essentially facilitated contractions differing from those of the sphincter of *Calliactis* only in their much longer time scale. But, as we shall show in this paper, there are in fact important differences between the contraction of the slow parietal and circular muscle systems on the one hand, and that of typically facilitated fast muscles on the other. Moreover, these slow muscles can show prolonged complex co-ordinated activity which seems to be spontaneous; that is, it is inherent in the animal and not the direct consequence of individual external stimuli (Batham & Pantin, 1950*b, c*). Further, their response to stimulation has more in common with this spontaneous activity than with a simple and direct contractile response to a stimulus. Accordingly, it was necessary to re-examine these phenomena. We choose particularly the slow responses of *M. senile* for this purpose.

METHODS

Anemones for experiments were collected carefully without rupturing the pedal disks, and placed in tanks on small stones or squares of ground glass which had for some hours previously been soaked in sea water. Plastic or celluloid collars helped

keep the anemones from drifting off stones or glass before their attachment, which may take a day or so. Anaesthetization with equal parts of 7½% $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ and sea water for about half an hour usually preceded the sewing in of recording threads—each a double loop of thick cotton. The anemones were then left to recover in sea water for a few hours or overnight. The specimens should be starved for at least 3 days prior to low-frequency stimulation tests. Otherwise, the peristaltic activity associated with ejection of food remains may modify reactions in a way subsequently to be described.

The actinians were stimulated by condenser shocks applied at various frequencies through non-polarizable Ag/AgCl electrodes in the manner described by Hall & Pantin (1937). Movements were recorded kymographically with long light isotonic levers attached to the animal by threads sewn into it.

Experiments were made not only on intact animals but also on various preparations of isolated parts. In making such preparations it is essential that all operations should be quickly and deftly carried out; otherwise there is great and prolonged contracture and secretion of mucus, and the preparation becomes useless. The following plan proved best. Large specimens in good condition were placed in bowls of aerated sea water overnight. Next morning an equal amount of 7½% $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ was added to whichever bowl contained the most expanded anemone. 30–60 min. later about 50 ml. of the MgCl_2 solution was also pipetted into the coelenteron via the mouth. This was repeated several times in the next hour. At intervals the margin of the pedal disk was gently pinched to see if the retractor response was still obtainable. Half an hour after it vanished the anemone was lifted out and the operation performed. The prepared piece of tissue was at first gently stretched with the fingers. One end of the strip or loop was then attached by a soft string and pins to a weighted cork base in a large bowl of aerated sea water. A thread from the other end of the preparation hung over the edge of the bowl and was attached to a plasticine weight heavy enough to raise the tissue in the water.

The tissue was then left for 3 or 4 hr. to recover, though the threshold for electrical stimuli might remain rather high and erratic for a further hour or two. By this time, in good preparations, the threshold and the response were about the same as in intact anemones.

FACILITATION AND THE SLOW RESPONSE IN *METRIDIUM*

Both the slow responses of the parietal muscles and the rapid facilitated contraction of the retractor can be elicited by electrical stimulation of any part of the column. In both, the response is not locally confined to the region stimulated but takes place round the whole column of the anemone; that is, there is through-conduction. In both also there is a threshold below which no response can be elicited; and the threshold for the response in both systems of muscle is precisely the same. It therefore appears that each system of muscles, the parietal and the retractor, can be excited by way of the same through-conduction system.

But these common features do not imply identity of the mechanism of excitation of the muscles themselves in the two systems, and further inspection shows essential

differences. The parietal contraction is much slower than that of the retractor. It is also quite smooth and sigmoid, showing none of the sharp step-like increments corresponding to each stimulus, which are characteristic of the retractor's facilitated response. At first sight this might perhaps be attributed to the slower mechanical properties of the muscle. A higher 'viscosity' might smooth out the step-like increments. But that will not account for the extraordinarily long delay between the stimulus and the moment when these slow responses begin. If a quiet and intact *Metridium* is given a regular series of shocks, the ensuing parietal response regularly begins at about the seventh shock (Fig. 1*b*). This holds over a wide range of frequencies, from about 1 stimulus every 2 sec. to 1 every 15 sec.

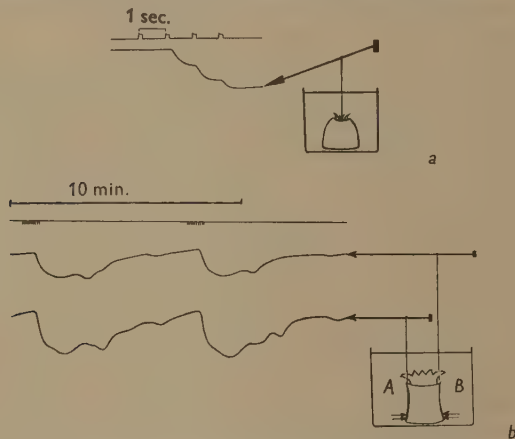


Fig. 1. (a) *Metridium* Retractor response to 4 condenser shocks. (b) Two parietal responses to series of 10 shocks at 5 sec. interval.

If the stimulus series be short, another important feature is apparent. There is an enormous latent period; so that the response not only shows no direct relation to the individual shocks, but may even take place long after the stimulus series has ceased.

These peculiarities of the parietal contraction are shown even more clearly by the very slow circular muscle of the column. Fig. 2*a* shows the responses of a ring preparation of circular muscle, 4 cm. in diameter. The ring was left attached to a longitudinal strip of body wall, on which the electrodes were placed 1.5 cm. from the ring. Stimulation was thus indirect. The sigmoid character of the contractions makes their exact beginning hard to determine, but in this case it is evident that the latent period exceeds 100 sec. In contrast, the latent period of each step of the retractor response (Fig. 1*a*) is only some 50–80 msec.; and even this is made up chiefly of conduction time in the through-conduction system. It is the enormous latent periods which differentiate the slow contractions most completely from facilitated ones.

These long latent periods of the parietal and circular muscles are not due to slow conduction up the column of the anemone. The through-conduction system which

excites these muscles conducts at a high speed, as can be seen at once by the promptness of the facilitated response of the retractor if the frequency of stimulation is raised. Hence the delay in the slow muscles must be peripheral. In the intact animal, different sectors of the parietal muscle system respond with periods of delay which differ in an arbitrary way from sector to sector and bear no relation to the site of stimulation. We shall discuss these parietal delays later.

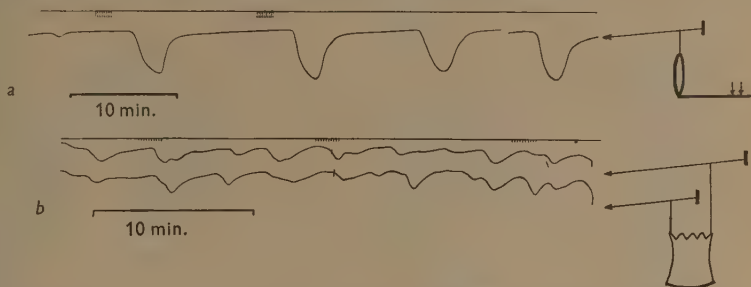


Fig. 2. (a) Ring of circular muscle from half way up column. Spontaneous contractions, and responses to 10 shocks at 10 sec. interval. Electrodes on tongue of body wall, 1.5 cm. from muscle ring. Note regular latent period of about 110 sec. (b) Parietal responses of intact active *Metridium* to series of 10 shocks at 10 sec.

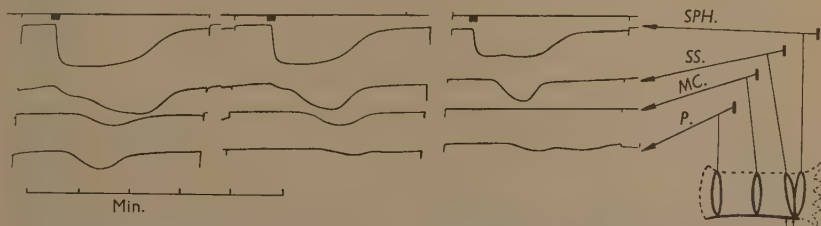


Fig. 3. Rings of circular muscle. Electrodes on subsphincter ring. Responses to series of 10 shocks at 1 sec. SPH., sphincter; SS., subsphincter; MC., mid-column; P., pedal.

The peripheral character of these long latent periods is even more clearly shown in preparations of the circular muscle. A preparation is made in which the column of the anemone is cut horizontally at various levels, to give a number of rings of the column wall. These rings are left in contact with a longitudinal strip of the column. We thus have a preparation consisting of a connected series of rings of the circular muscle sheet of the column, from the region of the marginal sphincter to near the pedal edge (as in Fig. 3).

Electrical stimulation of such a preparation may cause all the muscle rings to contract. If the frequency of stimulation is high, as in Fig. 3, a rapid facilitated response can be evoked from any muscle rings which include a portion of the marginal sphincter. The promptness of this response, whatever the point of stimulation, shows the high speed of conduction in the preparation.

But, in addition, every muscle ring shows a slow response with its characteristic delay. In the first record of Fig. 3, the first ring to respond, after the facilitated contraction of the sphincter, is the pedal loop (15 sec. after the last stimulus). The next is the mid-column ring (26 sec.), whilst the slow main contraction of the sub-sphincter begins some 29 sec. after the last shock; though the preceding small facilitated response makes the exact onset hard to determine.

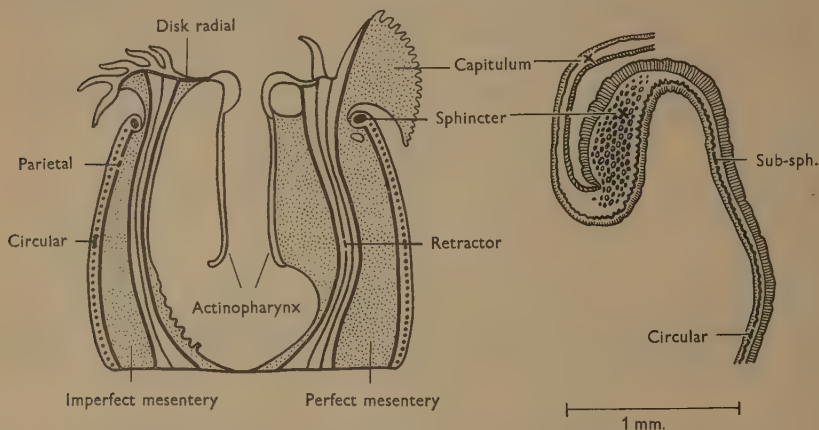


Fig. 4. Diagrams illustrating the musculature of *Metridium*, and the anatomical position of circular muscle regions.

As in the responses of different sectors of the parietal muscle system, the latent periods of these rings of circular muscle vary, and the order in which the rings contract bears no relation to the site of the stimulus. In both the first two records of Fig. 3 the electrodes were placed at the oral end of the preparation (near the subsphincter); but the order of contraction of the rings is not the same. And though it so happens that in the second record the muscle loops contract in an orderly succession from the electrodes, nevertheless when those were placed at the opposite end near the pedal loop, as in the third record, the first loop to receive the excitation was actually the last to give its slow response.

Evidently, in both circular and parietal muscles, excitation by way of the through-conduction system is followed by prolonged delay; a delay about a thousand times that in facilitated responses, and one which occurs locally in the neighbourhood of the responding muscle rather than in the through-conduction system.

NUMBER AND FREQUENCY OF STIMULI

In animals or preparations showing little spontaneous activity it is possible to study the relation of the size of the slow responses to the number and frequency of stimuli. Here also the relation differs from that of simple facilitation. Preparations have an advantage over the intact animal because the response is not complicated by the

contractions of several different muscle systems; and consequently the response to a wide range of frequencies can be clearly studied.

In a typical experiment, a ring of quiescent circular muscle cut from just below the marginal sphincter was stimulated by electrodes placed on an attached tongue of body-wall tissue. As in the step-like facilitated contraction of the retractor muscle, the size of the smooth slow response of this 'subsphincter' ring of circular muscle was more or less constant over a certain range of frequencies; diminishing when these were still higher (cf. Hall & Pantin, 1937). The range of frequencies over which the subsphincter gave a fairly constant response was, however, surprisingly large—from 1 stimulus in $\frac{1}{4}$ sec. to 1 in 20 sec. And below this, instead of the regular progressive decline characteristic of facilitation, the response merely became irregular. At frequencies of 1 stimulus in 40, 60 or 80 sec. there was a complex succession of contractions and relaxations which could not be related to individual stimuli, and which might have been no more than an increase in 'spontaneous activity'.

The number of stimuli required to produce a detectable response in these slow muscles is generally much larger than that needed for a facilitated contraction. Though the number needed for a parietal response varies from one animal to another, at least 3 or 4 stimuli are usually required. Beyond this the response tends to increase with the number of stimuli, but above 15 or 20 stimuli it gets no bigger, and long continued stimulation leads to irregular contractions and relaxations; whereas in a facilitated contraction each stimulus of a series adds a contraction step till there is a maintained tetanus.

A further, and important, feature of the slow contractions is their frequent variability, which contrasts with the machine-like regularity of facilitated contractions. It is most evident if the animal, or tissue preparation, is undergoing much spontaneous activity. Fig. 2*b* shows the parietal response from two sides of an intact animal which, unlike that used for Fig. 1*b*, showed great spontaneous activity. Here the spontaneous activity so greatly modifies the response that occasionally it is hard to tell whether contractions which follow stimulation are in fact caused by it. Even in an animal giving regular parietal responses, these may suddenly diminish and even fail after long experimentation. This is not through failure to excite the nervous system, for a rapid pair of stimuli will still evoke a normal facilitated response of the retractor muscle.

The slow contractions thus differ from typically facilitated ones. In the latter, the response to a number of shocks is chiefly a mechanical summation of unit steps. The contraction of the parietal and circular muscle systems is not of this kind. Here, the successive shocks seem to cause a state of excitation in the muscle which, if it reaches a sufficient intensity, is followed after a considerable delay by a smooth contraction. Unlike a facilitated one, the size of a slow contraction does not decrease in a simple way with decreasing frequency of stimulation, and, moreover, often shows considerable variability which seems to be related to spontaneous activity in the responding muscle.

OF QUICK AND SLOW CONTRACTION

The existence of two distinct kinds of contraction in actinians raises the question of whether they possess muscles of more than one sort. They show no histological evidence of two clearly distinct kinds of muscle fibre (Batham & Pantin, 1951). All are unstriated; though the fibres in different parts of the body do vary considerably in length, from 20 to 50 μ in the circular muscle sheet and in the radial muscle of the mesenteries to over 1 mm. in the mesenteric retractors of *Metridium*. But even the retractors are organized in the same way as the general muscle sheets: they are simply hypertrophied regions of a widespread muscular network. All we can say is that specific effectors, like the retractors of *Metridium* and the sphincter of *Calliactis*, which give powerful facilitated contractions, tend to possess large, long fibres. We cannot even say that quick facilitated responses are to be found only in such anatomically differentiated effectors, for, as we shall see, they can be obtained from the radial musculature of the oral disk.

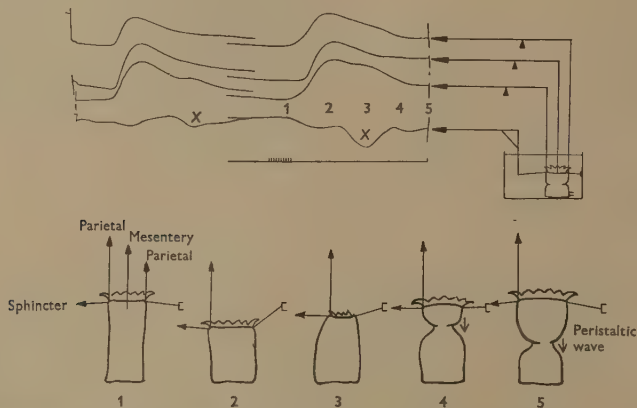


Fig. 5. Spontaneous mesenteric parietal-circular sequence, and the same induced by a series of 10 shocks at 3 sec. interval. Diagrams illustrate successive shapes assumed.

While there is no evidence that the muscle fibres of actinians fall into two distinct kinds, with different kinds of contraction, there is evidence that in some muscles the self-same muscle can contract in both the quick and the slow way. The mesenteric retractors in *Metridium* give a rapid facilitated response to rapid stimuli. But they can also show typical slow responses. Fig. 5 shows such a smooth delayed mesenteric contraction (second lever down) in the response following a low-frequency stimulus; the contraction begins well after the stimulus is over. In *Calliactis* such a slow delayed contraction is the most obvious element of the mesenteric retractor response.

If the retractor of *Metridium* could execute only quick facilitated contractions, it should remain extended or become bent and buckled when an extended animal

slowly shortens in this way. This does not happen. And when whole mesenteries are removed from the animal and observed under the microscope it is clear that all the fibres of the muscle sheet, including the retractor, can slowly and smoothly contract. It is hard to account for these phenomena except by supposing that the same fibres can contract in both ways.

The mesenteric retractor is not the only muscle which seems to contract in more than one way. Hall & Pantin (1937), working on intact *Metridium*, considered the marginal sphincter to be a very slow muscle, which however was facilitated by stimuli of about the same range of frequency as the rapid retractor. But our present work has thrown a different light on the matter. Owing to the proximity of the powerful retractors, the behaviour of the sphincter is not easy to analyse in the

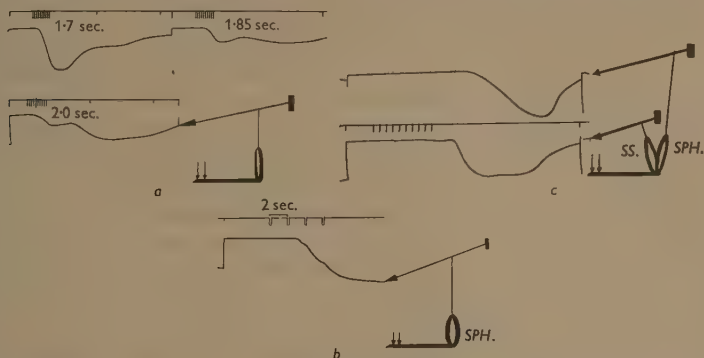


Fig. 6. (a) Responses of muscle ring including sphincter and neighbouring tissue. Electrodes on pedal end of tongue of body wall. (b) Same preparation on faster drum. (c) Responses of connected muscle rings of sphincter and subsphincter of 10 shocks at 10 sec.

intact animal. But this difficulty can be overcome by using preparations like those already described in which the sphincter and various parts of the circular musculature were partially separated as isolated rings of tissue. These rings of tissue remained connected along one side by a strip of body wall on which the electrodes could be placed.

Fig. 4 shows the anatomical relations of the sphincter muscle, the circular muscle sheet and the physiologically specialized region of it, the 'subsphincter'. It is from the subsphincter that peristaltic waves usually begin.

If a ring of tissue is isolated, including the sphincter, the response to rapid stimuli is distinctly double. This can be seen in Fig. 6a for shocks at frequencies of 1.7, 1.85 and 2 sec. There is one component which clearly facilitates with step-like contractions, as shown in the faster record of Fig. 6b. This resembles facilitation of the retractor of *Metridium* or of the sphincter itself in *Calliactis*. The only obvious difference is that the facilitated sphincter responses of *Metridium* sometimes require a number of preliminary stimuli before the contraction steps begin (in Fig. 3 it

begins at the 5th shock), whilst at other times they may show some degree of permanent facilitation, so that a single stimulus causes a response. In records from the intact *Metridium* this facilitated sphincter response tends to be lost in the disturbance due to the great facilitated retractor responses at the same range of frequency.

As in the retractor, the facilitated contraction of the sphincter of *Metridium* vanishes as frequencies of one shock in 3 sec. are approached (at 16–17° C.). But on approaching this frequency, the second component of the response ceases to be masked by the first, and becomes increasingly evident. In contrast with the facilitated contraction, this component has all the characteristics of the slow, sigmoid type with long latent period.

The interpretation of this double contractile response of the sphincter region is not easy. The marginal sphincter itself is anatomically differentiated within the mesogloea from the circular muscle layer from which it is derived. It is not possible to make a preparation including the sphincter but completely excluding the underlying circular muscle layer of the body wall. In ring preparations of the body wall, all parts can give the slow delayed contraction. But only those which include part of the marginal sphincter can also give a clear facilitated response (Fig. 3): all other parts give the slow contraction alone. We can thus attribute the fast, facilitated responses to the sphincter. The slow contractions of ring preparations which include the sphincter may partly be due to that portion of the general circular muscle layer inevitably included in the preparation. Nevertheless, there is some evidence that the sphincter itself contributes to the slow delayed contraction. Direct observation shows that when the sphincter region slowly contracts, all parts are involved in the process; we do not find an extended inactive sphincter bending and buckling as the innermost region of the preparation contracts.

Quick facilitated contractions can also be obtained from the radial muscle system of the oral disk. A sector of the disk of a large *Metridium* was cut out; its tentacular edge was then pinned down, and electrodes placed upon it. The radial contractions were recorded from a thread attached to the pharyngeal edge of the cut sector. Such preparations showed small but typical responses to every shock after the first of the series, provided the stimulation frequency was of the same range as that effective for the retractor (less than 1 per 2 sec.). Pantin (1935*a*) noted that in *Calliactis* even a single stimulus could cause a twitch of the tentacles owing to excitation of radial musculature. Directly facilitated contractions of the radial muscle can be obtained in this way from stimulation of either the oral region or the distal edge of the disk. In fact, in radial directions the disk shows through-conduction. This radial through-conduction contrasts strongly with the localized conduction laterally round the circumference of the disk. Early experiments showed (Pantin, 1935*a*) that in this direction the response of the disk to stimuli shows an apparent decrement as it is transmitted round the circumference; owing to what was termed 'interneural facilitation'.

It is important to notice that this localization of the response is restricted to circumferential conduction, and that radially there is 'linear through-conduction'.

This may be contrasted with the total through-conduction in every direction of the retractor system both over the whole of each mesentery and from one mesentery to another. But radially, the facilitated disk responses are quite comparable to facilitated responses of the retractor. And like those, in addition to simple facilitation, slow delayed responses were also obtained to stimuli of low frequency, between 1 in 5 and 1 in 20 sec. Unfortunately we cannot tell certainly whether the quick facilitated contraction and the slow responses were due to two different muscle systems or to one alone: for there are not only powerful radial muscles in the ectoderm of the disk, but also some endodermal radial fibres along the insertion of the mesenteries. But again we may note that slow contraction of the disk does not cause buckling as would be expected if the ectodermal muscle took no part in it.

All parts of the actinian muscle system can give the slow type of response. Some parts, such as the undifferentiated circular muscle of the column and the parietal muscles, appear to give none other. There is no evidence that any part can give rapid facilitated contractions alone. But some parts of the animal, like the radial muscle of the disk and the anatomically differentiated retractors and marginal sphincter, show both the quick facilitated contractions and the slow type as well. There is no evidence of two sharply distinct histological types of muscle fibre to account for this. On the other hand, the direct observation of the retractor and of the sphincter indicates that when these slowly shorten all parts contract and none remains passively extended. Though nothing but direct observation of the contractions of individual living muscle fibres can be conclusive in such a case, it is hard to explain the phenomena unless the same muscle fibres can contract in both fashions. The phenomena recall in some ways the two kinds of contraction which can be elicited from some Crustacean muscles (Wiersma, 1952): though in the anemones, there is at present no evidence of multiple innervation of the muscles.

COMPLEXITY OF THE SLOW RESPONSES

An important feature of the slow response is that in the intact animal it is generally not a simple contraction of a single muscular system, but a co-ordinated sequence of activities. Fig. 1*b* shows two successive responses of an intact *Metridium* to a series of 10 shocks at 5 sec. interval. In this anemone, each such series of shocks caused a parietal contraction with great regularity; but this was followed by prolonged activity.

An initial parietal contraction is usually followed by a slow contraction of the marginal sphincter, and this in turn by a peristaltic wave. This can be seen in Fig. 5, which shows the response of an intact *Metridium* to a series of 10 shocks at 3 sec. interval. It is recorded from two points on the parietal wall, from the mesenteries and laterally from the marginal sphincter. The parietals lead, and are followed by the mesenteries: after an interval the marginal sphincter contracts, and this initiates a peristaltic wave. Such sequences are also characteristic of spontaneous activity (Batham & Pantin, 1950*b*; Pantin, 1952). A spontaneous movement of the column is recorded in Fig. 5 previous to the response to stimulation. The spontaneous contraction and the response to stimulation are essentially the same.

The similarity between spontaneous contractions and responses to low frequency stimulation extends even to details. When a parietal contraction takes place during spontaneous activity, the different sectors of the parietal wall do not all contract simultaneously and to the same extent. One side acts as 'leader'; and this may be at any point round the disk, and is as often seen between two of the recording strings as immediately beneath one of them. The remaining sectors begin their contractions in some cases as long as a minute after the leader and do so in an irregular order;

Table 1

		Stim.- leader interval						
9.08 p.m.	—	—	B	A	C	C	A	Sp.
			—	3	10	68	73	78
9.20	—	—	B	C	A	Sp.		
			—	—	—	—		
9.24	Stim.	35	B	C	A	Sp.		
			—	5	12	45		
9.33	—	—	B	A	C	Sp.		
			—	10	13	45		
9.38	Stim.	35	B	C	A	Sp.		
			—	5	10	50		
9.51	—	—	B	A	C	C*		
			—	28	30	90		
9.58	—	—	B	C	A	Sp.		
			—	10	13	40		
10.06	Stim.	40	B	C	A			
			—	5	5			
10.15	—	—	B	?	?			
			—	—	—			
10.19	Stim.	35	B	C	A	Sp.		
			—	10	10	35		
10.27	—	—	B	C	A	Sp.		
			—	5	8	35		
10.33	Stim.	40	B†	C†	C†	B†	A	Sp.
			—	8	35	38	40	50
10.37	Stim.	25	B	C	A	Sp.		
			—	15	25	75		

* Small circular contraction in progress in column.

† Very slight contraction.

‡ Very large contraction.

though this order may be repeated with each contraction over long periods (Batham & Pantin, 1950a). The parietal response to electrical stimulation is of exactly the same peculiar character. Moreover, the responses to stimulation tend to call into activity the same leading sector, and the other sectors respond in the same arbitrary sequence that is seen in a spontaneous response in the same animal. Thus, a specimen was from time to time giving spontaneous parietal contractions; and occasionally between these, stimuli of 10 shocks at 5 sec. interval were given. The parietal contraction was recorded from three different sectors of the body wall. The subsequent contraction of the marginal sphincter was also recorded. Table 1 shows the sequences observed. The parietal sectors are arbitrarily lettered A, B and C,

and the sphincter Sp. The delay of each sector after the leading parietal is shown in seconds. The responses to stimuli are marked 'Stim.': and in these the time between the first shock and the response of the leading parietal is also shown in seconds. Contraction sequences not marked 'Stim.' are spontaneous.

It will be seen that parietal sector B leads throughout, and that the sequence of the other parts and their delay times follow essentially the same pattern whether we are concerned with a spontaneous contraction or response to stimulation. We might in fact almost say that a stimulus seems to unloose a pattern of 'spontaneous' activity in the parietal system rather than cause a simple contraction of a single muscle. Such a system is not only distinct from simple facilitation, but also presents an obvious parallel to autonomic excitation of the semi-autonomous viscera and vascular systems of the vertebrates.

RECIPROCAL INHIBITION

The complexity of slow responses implies interaction between the muscular components of the column. Occasionally, though by no means invariably, there is clear evidence that a contraction present in one set of muscles actually inhibits the

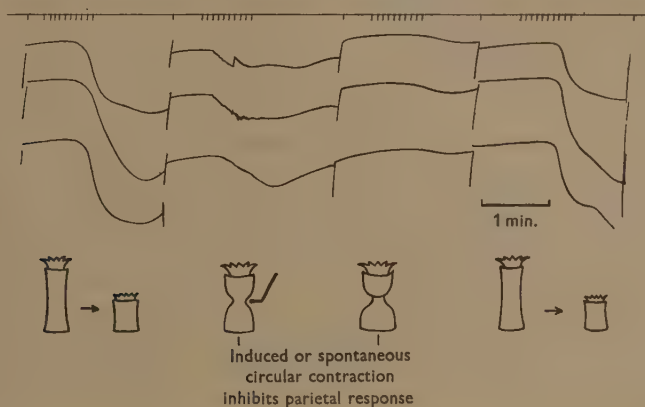


Fig. 7. Inhibition of parietal contractions by spontaneous circular contractions and by contractions induced by mechanical stimuli.

response to stimulation of the opposing set. In the column, the action of the parietal muscles is mechanically opposed by that of the circulars. Now it is possible directly to excite a local contraction of the circular muscle by local mechanical stimulation of the column. Fig. 7 shows the influence of such local contractions of the circular muscle on the parietal response. The anemone was giving strong and fairly regular parietal responses to series of 10 condenser shocks at 5 sec. interval. The first contraction of the series was given by the resting anemone when stimulated. During the second series of stimuli, a constriction of the circular muscle was induced by gently stroking the body wall with a rounded glass point: the contraction of the

parietal is very greatly diminished. During the third series of shocks a large spontaneous circular muscle contraction developed just above the pedal disk: the parietal contraction is almost abolished. The fourth series of shocks was unaccompanied by any circular activity; and the parietal response is very large. After the record illustrated, a fifth series of shocks again showed the effect of a mechanically induced circular contraction in reducing the parietal response; and a last series of shocks again showed the large uninhibited parietal contraction.

In this experiment, the circular muscle contractions, whether spontaneous or induced, extended right round the column so that every sector was affected. But it sometimes happens that mechanical stimulation of the column wall induces only a local contraction of the circular muscle which does not extend right round. When this happens, electrical stimulation may fail to elicit any parietal response in the sector with the localized contraction of the circular muscle, but it still causes a full parietal response in sectors on the opposite side: the animal thus bends away from the mechanical stimulus. This localization of parietal inhibition shows clearly that the failure to respond cannot be a simple mechanical effect, such as might follow a general rise of coelenteric pressure consequent on circular contraction.

Just as there is evidence of inhibition of the parietals by the circulars, so also there is occasional evidence of the converse. Sometimes electrical excitation of the parietal will cause a concurrently induced circular contraction to fade away; the parietal response apparently inhibiting the circular contraction. In one experiment a series of shocks at 10 sec. interval induced a prolonged state of parietal contraction which endured for some time after the stimulus. During this period the 'spontaneous' contractions at the circular muscle were almost completely inhibited.

Fairly often there appears to be a reciprocal relation between parietal and circular responses, in that those animals which give strong parietal contractions often give weak circular ones, and vice versa. But such a relation is not invariable, and sometimes a stimulus leads to simultaneous contraction of both parietal and circular muscles with consequent expulsion of sea water; as in the 'withering' reaction (Batham & Pantin, 1950*a*). Nevertheless, we may conclude that reciprocal inhibition is a factor, and perhaps a very important one, in the slow parietal-circular sequence.

CO-ORDINATION OF THE PARIETAL MUSCLE SYSTEM

It is evident that, both in the response to low-frequency electrical stimulation and in the essentially similar spontaneous movements of the column, the parietal-circular sequence involves co-ordination. The first phase of these movements is a contraction of the whole parietal system of muscle. This itself requires co-ordination, for the parietals are also capable of purely local contractions. Local parietal contractions are produced by certain specific stimuli. Light, even in the pure white variety of *Metridium*, causes them. Fig. 8 shows the response of such an animal to lateral illumination of the middle of the column for 30 sec. at about 5000 metre-candles. The animal was in a dark vessel for 2 hr. or more before the experiment. Recording threads were taken from the lighted and unlit sides. The local parietal contraction

to such illumination starts some 15-25 sec. after illumination begins. It never reaches maximal extent. After prolonged exposure (5-10 min.) there may occur a secondary, greater, and more general contraction involving retraction of the capitulum and constriction of the sphincter. Apart from the possible general excitation through spread of light during prolonged illumination, this secondary response differs from the primary local one in that it can be antagonized by recent feeding, particularly the sphincter contraction.

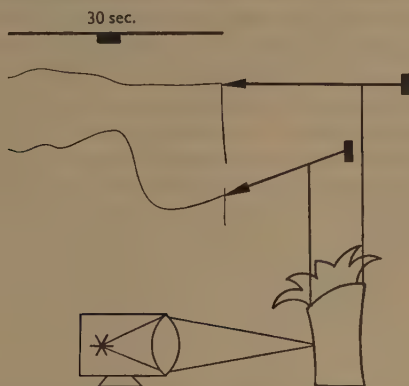


Fig. 8. Local parietal response to local light stimulus of 30 sec.

In *Metridium*, local parietal contraction cannot be initiated by mechanical stimuli. In some other species, local mechanical stimulation can cause striking local parietal responses. In a Brazilian *Bunodactis* (sp. ?), electrical stimulation causes slow symmetrical activation of the parietal musculature, but local gentle mechanical stimuli cause remarkably rapid and localized parietal contractions (Pantin & Vianna Dias, 1952).

The parietals are separate cords of muscle, not connected by a common muscle sheet. The above experiments show that these can be excited locally and independently by specific stimuli. When electrical stimulation causes all the parietal muscles to contract, it is exciting them by a different path, the through-conduction system. It is, in fact, the excitation of this system which co-ordinates the response of the separate parietal elements.

Excitation of the through-conduction system thus plays an essential part in initiating the parietal-circular co-ordinated response to electrical stimulation. Now we have seen that there is a fundamental similarity between this response to electrical stimulation and the spontaneous co-ordinated parietal-circular sequence (Fig. 5 and Table 1). This similarity suggests that, just as in electrical stimulation, these spontaneous contractions are initiated by occasional low-frequency nervous impulses, in this case of natural origin, traversing the through-conduction system. By analogy with the results of electrical stimulation, we should expect the larger

spontaneous contractions to coincide with bursts of several impulses at a frequency of the order of one impulse per 5 or 10 sec.

There is no doubt that impulses do pass occasionally over the through-conduction system in apparently unstimulated animals. Very occasionally even unstimulated intact animals will be seen to give a 'spontaneous' contraction of the retractors; a response which will only occur if two impulses pass over the through-conduction system within about 2 sec. of each other. Spontaneous contractions of the retractor are very rare, and the hypothesis we are considering would not demand the presence of impulses in the through-conduction system at the relatively high frequency required to produce them. However, the presence of spontaneous impulses even at very low frequency should be brought to light in another way. If a single electric stimulus happens to be sent into the through-conduction system within 2 sec. before or after a spontaneous impulse, the resulting pair of impulses (one generated spontaneously, the other set up by the single stimulus) will be sufficiently close together to cause a quick contraction of the retractor. Such an effect can in fact be observed. It is possible to gauge the natural frequency of spontaneous impulses, by comparing the number of times which a single electric shock happens to call forth a retractor response, with the number of times it happens not to. Since a retractor response will occur if a stimulus happens to fall within about ± 2 sec. of a 'spontaneous' impulse, it may be said that each stimulus 'investigates' the presence of such an impulse over a 4 sec. period. It follows that if 1 in n shocks succeeds in eliciting a response, there is about 1 spontaneous impulse occurring every $4n$ sec.

In a total of 3241 single shocks, given to twelve different *Metridium*, 42 caused a retractor response. This corresponds to an average of about 1 spontaneous impulse for every 80 shocks: and, if each shock would reveal a spontaneous impulse within ± 2 sec., this implies an average frequency of 1 spontaneous impulse every 5 min. Individual animals vary greatly in the apparent frequency of 'spontaneous' impulses. In those we have studied the average frequency varied from 1 such impulse every 2 min. to 1 in 12 min. Ross (1952) has published figures (his table 1) which would indicate from 1 impulse every 7 min. to 1 every 70 min. These values are probably too low for normal resting anemones, for Ross was continuously stimulating his animals for 24 hr. at frequencies of 1 shock in 5 sec. to 1 in 30 sec.; and continual low-frequency stimulation depresses spontaneous activity (cf. Batham & Pantin, 1950*b*, Fig. 5). Our anemones, also, were subjected to fairly frequent stimulation, though not continuously as in Ross's experiments. Even so, these experiments show that the frequency of spontaneous impulses is very low; moreover, these may include some 'after discharge' impulses generated by the stimulus itself.

Nevertheless, the animals chosen for stimulation experiments tend to be naturally quiet ones, and only a limited number of them show the co-ordinated parietal sequence. Moreover, such co-ordinated contractions only occur about every 10 min., so that occasional outbursts of impulses at long intervals are all that the phenomenon requires. The experiments certainly show that from time to time some spontaneous impulses traverse the through-conduction system.

Since any impulses causing parietal contractions must of necessity precede those contractions, it is not easy to devise a method of detecting their presence directly. However, highly suggestive phenomena have been seen in some preparations of circular muscle attached to a strip of body wall in the way described earlier. Fig. 9 shows an outburst of spontaneous activity of the very slow partly isolated subsphincter muscle. In this preparation the sphincter itself gave a small facilitated response even to a single shock. It will be seen from the record that the outbursts of slow activity in the subsphincter seem to be preceded by the appearance of occasional impulses registered by facilitated contractions in the partly isolated sphincter.

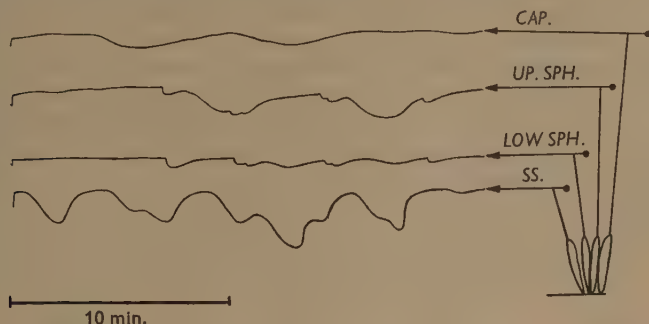


Fig. 9. Spontaneous activity in preparation of four connected strips of the column: capitulum (CAP.); upper (oral) half of sphincter (UP. SPH.); lower (distal) half of sphincter (LOW SPH.); subsphincter (SS.). Note simultaneous recording of spontaneous twitches in each portion of the sphincter; and the relation of slow contractions in subsphincter and in capitulum to these twitches.

CO-ORDINATION OF THE CIRCULAR MUSCLE SYSTEM

In the parietal-circular sequence, the slow parietal contraction is followed by a contraction of the subsphincter; and a wave of peristalsis then slowly passes down the column, taking several minutes to do so. This can be seen both in stimulated and in spontaneously active animals (cf. Batham & Pantin, 1950*b*, fig. 2). Such activity involves a co-ordinated sequence, not only between the parietal and circular muscle systems but also between the different parts of the circular muscle layer (Fig. 5).

Apart from the folding of the circular muscle sheet to give the marginal sphincter, the histological and anatomical organization of the whole circular muscle system of the column is of uniform character. But the various regions differ physiologically. Fig. 10 shows the spontaneous activity of partly separated rings of the sphincter, the subsphincter, the middle of the column and the pedal edge. These rings retained contact with a strip of parietal body wall as in the figure. There is a gradient of spontaneous activity, which is least in the region of the subsphincter and greatest near the pedal edge. This activity is mostly unco-ordinated; but as Fig. 10 shows, there are occasional contractions in which all four muscle strips act more or less together. These co-ordinated contractions are often preceded by a twitch of the

sphincter ring (Fig. 10), indicating that they are associated with occasional spontaneous impulses in the through-conduction system.

The co-ordination which is apparent when the four muscle rings contract together in this way is limited: for their precise sequence of contraction is neither orderly nor constant, though all contract within about 1 min. of each other. This is quite unlike what happens in the intact animal. In that case there is a higher degree of co-ordination. Whilst occasional circular contractions may start in various regions of the column, usually a contraction is followed by a peristaltic, or at times an anti-peristaltic, wave which brings about contraction of the parts of the circular muscle layer in a single, orderly succession. This peristaltic wave takes several minutes to traverse the column, so that the interval between a contraction of the subsphincter and that of the lowest parts of the circular muscle field reached by the peristaltic wave is far longer than the interval between the contractions of the partly separated muscle strips of Fig. 10. It is as though in the intact animal the peristaltic wave ensures a co-ordinated sequence of contraction down the whole muscle sheet, so that the contraction of each successive part is in some way held back until the peristaltic wave slowly reaches it in due order.

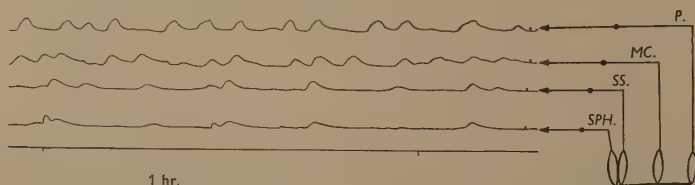


Fig. 10. Spontaneous contractions of connected rings of circular muscle from sphincter (SPH.), subsphincter (SS.), mid-column (MC.), and pedal region (P.). Note gradient of spontaneous activity and co-ordination of other rings with occasional twitches in sphincter.

This same contrast can be seen between the responses to stimulation in ring preparations and in intact animals. We have already illustrated those of such a ring preparation in Fig. 3. Here again, the rings of tissue do not contract in regular sequence; and despite the long latent period, the interval between their contractions is far less than the time required for the passage of the co-ordinating peristaltic wave in the intact animal. In the preparation illustrated in the first record of Fig. 3 the pedal circular muscle is responding only 15 sec. after the stimulus, whereas in the intact animal a peristaltic wave will not reach this region for several minutes. Secondly, individual loops, particularly near the pedal edge, may each show considerable spontaneous activity in which the other loops do not partake. Moreover, though all the muscle loops may respond to the stimulus they do not do so in due order. As in spontaneous activity, it seems as though in the intact animal the response of the circular muscle is temporarily inhibited and controlled; first so that the subsphincter follows the initial parietal contraction in an orderly way, and then so that the contraction of each part of the circular muscle sheet of the column is held back till the peristaltic wave reaches it.

Though a response of the muscle loops can be invoked by stimulation of the through-conduction system, the passage of the peristaltic wave in the intact animal cannot be due to transmission down that system: the conduction of the wave is much too slow and it shows an ordered sequence absent in the response of muscle loops. Whatever the system conducting peristalsis, it seems closely associated with the muscle sheet. Indeed this conducting system may perhaps be the muscle sheet itself, and not nervous. The circular muscle field of the column certainly provides a connected network of muscle fibres (Batham & Pantin, 1951). It is significant that, as Parker (1919) showed, even after Mg anaesthetization gentle mechanical stimulation of the column in *Metridium* can still give rise to a localized contraction of the circular muscle layer. And during Mg anaesthetization peristalsis of the circular muscle sheet is the last activity to disappear, failing after the through-conduction reflexes seem to have vanished. Moreover, peristaltic conduction is sensitive to deformation of the muscle layer. We have noted elsewhere (Batham & Pantin, 1950*b*) that slight local damage to the column of *Metridium* by the insertion of a thread may result in a local ring of diminished tone in the circular muscle. This region of low muscular tone acts as a barrier to the transmission of peristaltic waves. These facts do not permit us to conclude that peristaltic conduction is certainly muscular; though at present that is the simplest interpretation of them. It is, however, certain that peristalsis is not transmitted along the fast through-conduction system.

CONCLUSION

Our experiments show that when electrically stimulated at a very low frequency, the muscles of *Metridium* regularly respond, though in a manner different from rapid, facilitated responses. Though extensive, the response is very slow, with a very long latent period, and is variable. In the intact animal, it is not a single response of one muscle but a co-ordinated sequence of reciprocal antagonists. These complex responses to low-frequency stimulation are precisely similar to the rhythmic spontaneous parietal-circular sequences of contraction which occur naturally from time to time in the intact animal.

Analysis of the slow responses to stimulation can thus throw light on the mechanism of these spontaneous contractions. We have seen that the parietal muscle bands are excitable independently: though we do not yet know the pathway of this local excitation. We know also that these same muscle bands often execute local spontaneous activity independently of neighbouring parietals. Their activity may become co-ordinated, in the parietal-circular sequence. When it does so, one part of the parietal system acts as leader, and is followed, after some delay, by the other parts in an arbitrary order; and followed still later by a slow contraction of the sphincter region of the circular muscle, and this by a peristaltic wave. This complex and peculiar co-ordination of the parietals with the circular muscle system seen in spontaneous activity is of the same character as the response to low-frequency excitation of the through-conduction system.

We have also seen that there are occasional spontaneous impulses to be detected

in the through-conduction system; and that in circular muscle preparations impulses may be detected during spontaneous outbursts of slow contractions. It is reasonable therefore to suppose that the rhythmic spontaneous parietal-circular sequence is initiated by the more or less periodic discharge in the through-conduction system of a few impulses; these serving to co-ordinate the natural independent spontaneous activity of the separate parietal sectors. We do not know from whence these impulses arise; though they may come from the leading parietal sector itself.

After the co-ordinated parietal contraction there follows the slow contraction of the marginal sphincter, and this engenders a slow peristaltic wave. The conduction of this wave and the co-ordination of the successive parts of the circular muscle system which it entails are too slow to be due to transmission down the through-conduction system itself. There is some evidence suggesting that peristalsis is directly conducted in the muscle sheet.

SUMMARY

1. The very slow responses of the body wall of the actinian *Metridium senile* have been studied, both in intact animals and in partially isolated tissue preparations.

2. The slow responses to electrical stimulation differ from the rapid facilitated responses of the retractor muscle. There is an enormous latent period of peripheral origin. The contraction is sigmoid, and does not show the step-like character of the retractor response. The relation to frequency and number of electric stimuli also differs. The responses vary with the state of the animal.

3. Some tissues, such as the marginal sphincter region, give two distinct kinds of contraction: a quick facilitated response and a slow delayed response. There is no striking histological differentiation into two kinds of muscle fibres, though there is some anatomical differentiation into two regions.

4. In contrast with the total through-conduction to the sphincter and the retractor, the radial muscle of the disk shows radial 'linear through-conduction', the response remaining localized circumferentially. Nevertheless, in the disk also there are both quick and slow responses.

5. The slow responses in the intact animal are not simple contractions. They consist of a co-ordinated sequence in several muscles which may continue for many minutes. There is evidence of reciprocal inhibition between the circular and parietal muscles. The responses to the same stimulus vary greatly at different times.

6. The slow responses to electrical excitation show a detailed resemblance to the spontaneous contractions of the same muscle systems. The muscle systems excited are not passive, but spontaneously active systems.

7. The parietal muscle system can be excited to contract locally by specific stimuli. Electrical excitation excites always the whole parietal system. The co-ordinating system in the latter case is considered to be the through-conduction system. The complete identity of the spontaneous parietal-circular sequence with that resulting from electrical excitation indicates that the through-conduction system co-ordinates the spontaneous sequence as well as the response to the

stimulus. There is evidence of occasional impulses in the through-conduction system during spontaneous activity.

8. Partly isolated rings of the circular muscle in such a preparation of the body wall, connected by a strip of tissue, respond to electrical excitation. In contrast, in the intact animal the contractions of the parts of the circular muscle system are co-ordinated to give an exceedingly slow peristaltic or antiperistaltic wave.

9. The excitation system of the slow muscle resembles the visceral neuromuscular system of vertebrates rather than that of skeletal muscles such as the limb muscles.

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ACTIVE TRANSPORT OF WATER BY THE MALPIGHIAN TUBULES OF THE STICK INSECT, *DIXIPPUS MOROSUS* (ORTHOPTERA, PHASMIDAE)

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INTRODUCTION

In a recent study of excretion in insects (Ramsay, 1953*b*) it has been shown that in eight different genera potassium ion is actively transported across the wall of the Malpighian tubule from haemolymph to urine against an electro-chemical gradient. It is suggested that this process is likely to be of general occurrence among insects. In two of these genera it has further been shown (Ramsay, 1952, 1953*a*) that some of the potassium secreted into the urine is later reabsorbed into the haemolymph, so that there is a circulation of potassium within the insect's body; this again may well prove to be of general occurrence.

One is tempted to see this circulation as something which is fundamental to the process of urine formation in Malpighian tubules. It is easy to imagine that the secretion of potassium (together with some anion) into the tubule will set up an osmotic pressure which in its turn will promote a passive inward diffusion of water. According to this theory the secretion of potassium is, as it were, the prime mover in generating the flow of urine; and if the theory is true it follows that the osmotic pressure of the urine ($O.P._u$) should be equal to or greater than, but never less than, the osmotic pressure of the haemolymph ($O.P._h$).

Measurements of osmotic pressure reported in earlier work (Ramsay, 1950, 1951, 1952) show that, although the urine and the haemolymph are more or less isotonic, in certain cases $O.P._u < O.P._h$ by a small but apparently significant amount. These measurements taken at their face value are sufficient in themselves to disprove the theory; but before they are accepted it is necessary to point out that the conditions under which the fluids were collected were not entirely satisfactory where small differences of osmotic pressure are in question. In some experiments the insect was opened in a stream of moist air and some slight evaporation or condensation may have occurred. The circulation was seriously impaired as a result of the operation, and it is not impossible that haemolymph was collected from one region exposed to the air while the main part of the tubule lay in another region of the body unaffected by exposure. In other experiments haemolymph was obtained by tearing open the body of a small larva with gross damage to the tissues.

The present experiments were planned to avoid these disadvantages by making use of single Malpighian tubules removed from the body of the insect and set up in drops of haemolymph under liquid paraffin. In this way it is possible to ensure

that the sample taken is representative of the haemolymph surrounding the tubule, and differences in osmotic pressure can be interpreted with more confidence.

MATERIAL AND METHODS

The Malpighian tubules of the stick insect, *Dixippus morosus* (Orthoptera, Phasmidae), are particularly suitable for study. De Sinéty (1901) recognized three kinds of tubules. (1) 'Superior' tubules, opening at the annulus between midgut and hindgut, making a short forward loop and running back to end blindly close to the posterior region of the hindgut. The 'superior' tubules are of fairly uniform appearance throughout their length and are supplied by small branches of the tracheal system reaching them at various points. They are of relatively large diameter and the cells are transparent. (2) 'Inferior' tubules, opening at the annulus in pairs and running directly backwards. Their distal portions are dilated and filled with a milky fluid and they terminate in clumps of cells (cells of Sidorot) embedded in the fat body. Each tubule has its own trachea which accompanies it over most of its length. Over their non-dilated proximal portions the 'inferior' tubules have the same appearance as the 'superior' tubules. (3) 'Appendices of the midgut.' These appear to be very thin tubules opening separately into the midgut and running back over the hindgut.

De Sinéty further investigated the dilatations of the 'inferior' tubules and came to the conclusion that their contents were the carbonates of calcium and magnesium which accumulated in these dilatations throughout the insect's life. From these observations and from comparison of the superior and inferior tubules under the microscope it seems likely that the proximal portion of the 'inferior' tubule is a region where urine is produced, whereas the distal region is a 'kidney of accumulation' where alkaline earth carbonates are precipitated and fluid is reabsorbed. Some further evidence bearing upon this point was obtained during the course of the present work; it was found that potassium, secreted into the tubule in the proximal portion, passed back into the haemolymph in the distal portion. However, this circulation within the tubule is relatively slow, as is shown by the use of phenol red which is rapidly concentrated in the lumen of the proximal portion but only slowly moves into the distal portion. It seems that by far the greater part of the urine passes down the tubule and into the gut.

Both the 'superior' and 'inferior' tubules can be used in these investigations, but for purposes of removal from the body the 'inferior' tubules have the advantages of lying superficially and of being without many fine tracheal connexions. As will be pointed out in the next paragraph the speed with which dissection can be completed is an important consideration. For all the experiments reported in this paper 'inferior' tubules were used, either entire or with the distal portion ligatured off and cut away.

While it is not impossible to dissect out a Malpighian tubule from the body of a stick insect opened in air, the dissection is very much easier and quicker with the insect under saline. Unfortunately, the Malpighian tubules are very sensitive to

the composition of the medium in which they are immersed, and whereas other insect tissues survive well in physiological saline the Malpighian tubules show abnormal changes after a relatively short time (Wigglesworth, 1953). As described in the Appendix a dissecting fluid was made up in which the tubules could survive for a few hours as judged by their ability to concentrate phenol red. In practice the dissection could be completed in 20 min., after which the tubules were returned to haemolymph as described below.

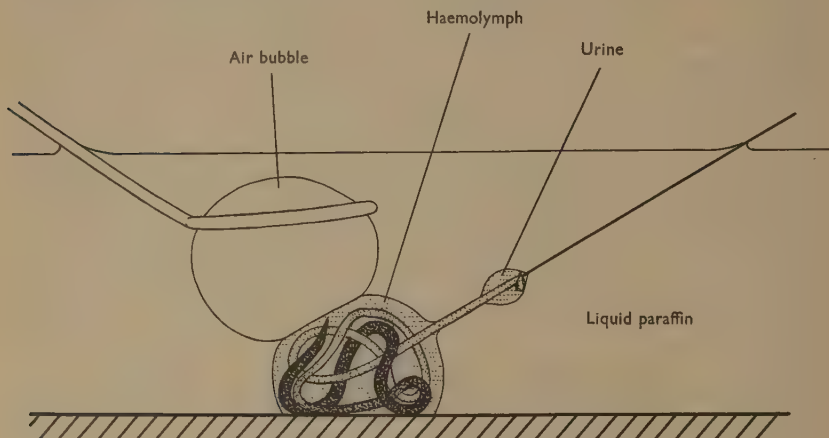


Fig. 1. For explanation see text.

The insect was pinned down ventral surface uppermost in a small dissecting dish. The abdomen was opened and as much haemolymph as possible was collected and stored under liquid paraffin in a varnished watch-glass. Dissecting fluid was then added to cover the insect and some half-dozen tubules were separated and cut free close to their openings into the gut. These tubules were removed by means of a pipette each to a separate varnished watch-glass containing liquid paraffin; here the dissecting fluid was sucked away and replaced with a drop of haemolymph. Tubules prepared in this way have remained alive for as much as 48 hr., exhibiting characteristic writhing movements and being able to concentrate phenol red.

Urine was collected in either of two ways: (1) the cut end of the tubule was seized with forceps and a pipette was inserted as described previously (Ramsay, 1953*b*), or (2) a fine silk thread was tied around the severed end of the tubule which was then drawn a short distance out of the drop of haemolymph and a cut was made close to the ligature to allow the urine to escape; the urine accumulated as a droplet around the ligature and did not tend to run back into the drop of haemolymph. Oxygen was provided from a bubble of air which was held in a loop of wire and pressed against the drop of haemolymph. The arrangement is shown in Fig. 1.

Osmotic pressure was determined by the depression of freezing-point method previously described (Ramsay, 1949), and is expressed as that concentration of

NaCl, in millimoles per litre of solution, which has the same freezing-point depression. Since the highest accuracy was called for, all (or nearly all) determinations were made in quadruplicate. The standard error of the mean of four determinations by this method is less than ± 0.7 mm/l. NaCl.

RESULTS

(a) *Relation between $O.P._h$ and $O.P._u$.* In the first series of experiments urine production was studied on single tubules each enclosed in a relatively large (4–5 cu.mm.) drop of haemolymph, it being assumed that the osmotic pressure of the haemolymph would not be significantly altered by the relatively small amount

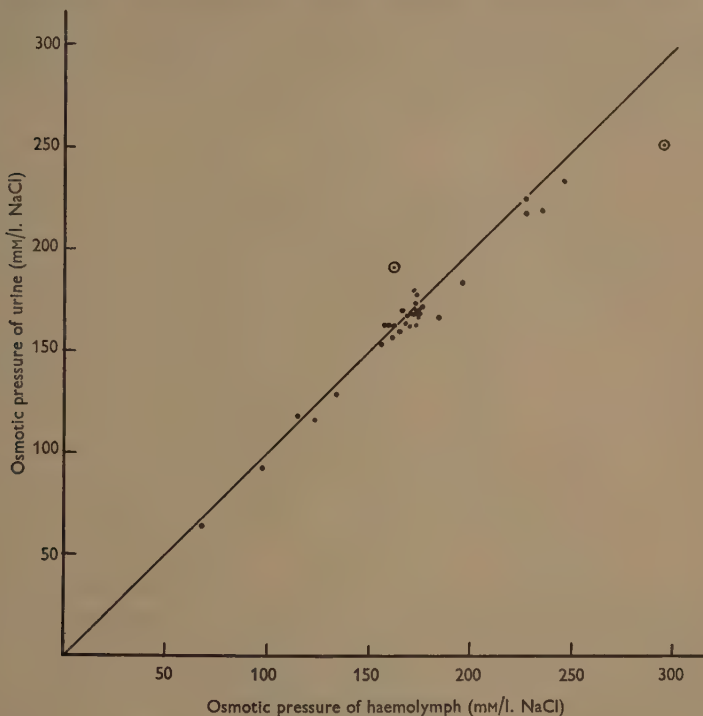


Fig. 2. For explanation see text.

of urine produced. In many of these experiments, after an initial collection of urine had been made and a sample of haemolymph taken, the remaining haemolymph was diluted with distilled water or its osmotic pressure was raised by the addition of sugar (see Appendix, component A) and further collections were made.

In Fig. 2, $O.P._u$ is plotted against $O.P._h$ for all the thirty-four observations made in this series of experiments. Apart from two cases, which are ringed in Fig. 2, the

difference $O.P._h - O.P._u$ is small; neglecting the two ringed observations the average value of the difference is 2.6 mm/l. Possibly this difference is not statistically significant in relation to the scatter of the observations, but that is an issue of secondary importance. What is important is that in a majority of cases $O.P._h$ exceeds $O.P._u$ by an amount which is significant in relation to the errors of analysis. It is impossible to doubt that the tubules can and often do produce hypotonic urine, and this is decisive in demanding the rejection of the theory of urine production which was outlined in the Introduction.

A second series of experiments was undertaken in which the distal portion of the tubule was ligatured off and cut away and the proximal portion only was enclosed in a relatively small (0.25–0.4 cu.mm.) drop of haemolymph. The main purpose of these experiments was to study the changes in the ionic composition of the haemolymph which were brought about by the activity of the tubule; it so happened that they provided confirmation of the conclusions drawn from the first series of experiments.

Table 1

t , time in minutes since start of experiment.
 $O.P._h$, osmotic pressure of haemolymph in mm/l. NaCl.
 $O.P._u$, osmotic pressure of urine in mm/l. NaCl.

Tubule no.	t	$O.P._h$	$O.P._u$	$O.P._h - O.P._u$
1	30	192	193	-1
	105	195	177	18
	195	194	176	18
	380	201	187	14
2	40	187	181	6
	115	181	164	17
	200	181	162	19
	385	188	162	26
3	55	193	181	12
	120	196	177	19
	205	196	180	16
	390	195	156	39

Urine was collected and a sample of haemolymph was taken at various times after the start of the experiment. The osmotic pressures of these samples are recorded in Table 1. The variations in $O.P._h$ during these experiments call for comment. During the first period the tubule is recovering in haemolymph from its previous immersion in dissecting fluid; its activities during this period and their effects upon the composition of the haemolymph may well be unrepresentative. During the second and third periods the tubule is probably working normally and $O.P._u$ is constant. By the end of the fourth period urine production has come to a standstill, and the tubule is no longer able to concentrate phenol red; this is quite probably the result of the changes (e.g. reduction of potassium) in the haemolymph which have been brought about by the activity of the tubule.

The figures in Table 1 show that apart from the first period of the experiment the urine is consistently hypotonic to the haemolymph and that $O.P._h - O.P._u$ is

many times greater than the average difference recorded in the first series of experiments in which large drops were used.

(b) *Rate of urine production.* Although it would appear to be a relatively simple matter to collect the urine which accumulates in a given time and measure its volume, this will not necessarily be an accurate measure of the rate of urine production. If one collects by means of a pipette the urine rises rapidly into the pipette, and the walls of the tubule collapse around the orifice. The pipette is withdrawn, the urine is ejected and the pipette must then be reinserted. It is scarcely practicable to insert a pipette into a collapsed tubule without its orifice becoming blocked; on the other hand, if one waits until the tubule is distended there is liable to be some loss during the process of penetration. If one collects the urine by the second method, described on p. 106, there is an immediate rush of urine after the tubule is cut, but the edges of the cut then come together and the pressure subsequently developed is not always sufficient to force them apart again. There is also the further difficulty that if the free outflow of urine is restricted and the tubule becomes over-distended there may be some inhibition of urine production (see below under Secretion Pressure). Measurements of the rate of urine production are therefore liable to considerable error.

Rough estimates of volume were made on the urine samples by measuring the diameter of the droplets as they rested under liquid paraffin upon the bottom of a watch-glass. The droplets were assumed to be spherical, an assumption which was manifestly untrue, but the errors arising from this approximation are small compared with the errors discussed in the previous paragraph.

The volumes of the urine samples whose osmotic pressures are recorded in Fig. 2 were measured in this way, and the times of collection being also recorded it was possible to calculate the corresponding rates of urine production. In Fig. 3 the rate of urine production is plotted against $O.P._h$ and shows an obvious tendency to increase with increasing dilution of the haemolymph; but the scatter is so great that no more accurate description of the relationship can usefully be given.

The length of a tubule is about 20 mm., of which the proximal portion (where it is assumed that the urine is produced) represents about 15 mm. The outer diameter of this region may be of the order of 120μ . From Fig. 3, 0.4×10^{-3} cu.mm./min. could be taken as a normal rate of urine production. From this data the rate of urine production per unit area of tubule surface works out as of the order of 0.07×10^{-3} cu.mm./sq.mm./min. or 0.42×10^{-3} c.c./sq.cm./hr.

(c) *Secretion pressure of urine.* It is commonly observed (e.g. Wigglesworth, 1931, 1933) that ligatured Malpighian tubules become distended but do not swell up so much as to burst, from which it may be assumed that urine production ceases after a certain degree of distension has been suffered. This in its turn implies that there is a maximum hydrostatic pressure against which urine can be secreted by the tubule. This matter was investigated in the following way.

As stated above, when a tubule is ligatured it swells to a certain limited extent. The diameter of the partially distended tubule is first measured, the tubule is cut so that the urine escapes with a rush and the tubule shrinks to its 'resting' diameter

which is then measured again. It is also possible artificially to distend the tubule by inflation. For this purpose a steeply tapered pipette is used, partially filled with a solution of 30 mM/l. NaCl + 150 mM/l. KCl which approximates to urine in composition at least as far as the cations are concerned. The pipette is connected to a water manometer and is thrust well into the tubule so that its taper seals the wound. The hydrostatic pressure necessary to produce varying degrees of distension of the tubule can then be measured, allowance being made for the capillary forces at the meniscus in the pipette.

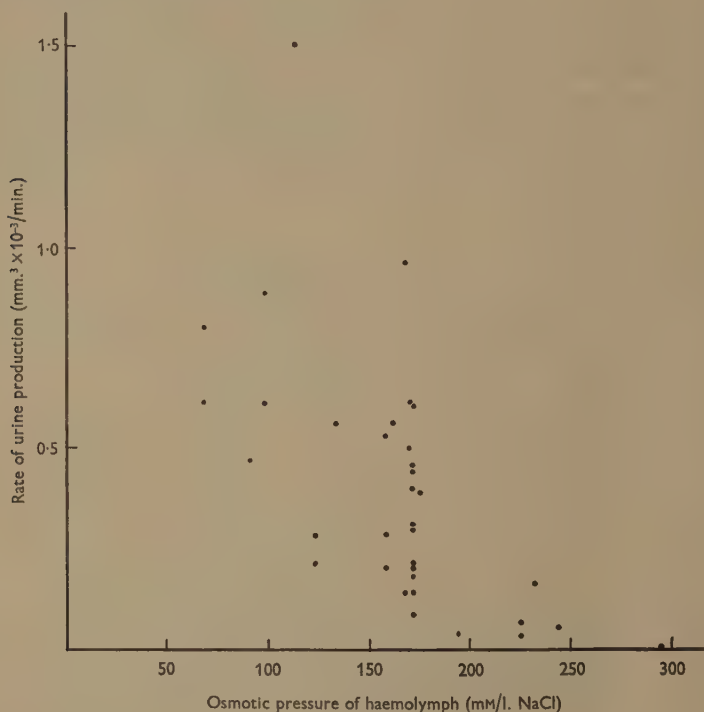


Fig. 3. For explanation see text.

Several tubules were investigated in this way. A pressure of 10–15 cm. H₂O was required to produce any visible distension of the tubule beyond its 'resting' diameter. With pressures increasing from 15 to 40 cm. H₂O the diameter of the tubule increased to about double the 'resting' diameter, and the contractile elements in the wall seemed to be unable to produce the writhing movements against this degree of turgor. Beyond a pressure of 40 cm. H₂O there was no further increase in diameter up to the pressure at which the seal gave way.

An increase in diameter to double the 'resting' diameter has only once been observed in a ligatured tubule; for the most part the distended diameter of a ligatured tubule is about 120–130% of the resting diameter, which would indicate a secretion pressure of the order of 20 cm. H_2O . Now it has been shown (Table 1) that urine can be produced against an osmotic gradient of some 20 mM/l. NaCl or more. In terms of hydrostatic pressure this is very many times greater than 20 cm. H_2O . There is therefore no question of the secretory mechanism being unable to overcome the hydrostatic pressure developed inside the ligatured tubule; if secretion fails this is more likely to be the result of the distortion suffered by the secretory cells as the tubule becomes distended.

It was interesting to observe that the fully distended tubule was unable to concentrate phenol red, a dye which the normal tubule concentrates very rapidly. This failure might have been due to the distension of the tubule *per se*, as suggested above, but it might also have been due to some metabolic disturbance resulting from the presence of the distending fluid in the lumen, since this fluid only roughly approximates to urine in composition. To put this to the test an experiment was carried out as follows. Phenol red was added to the haemolymph surrounding the fully distended tubule; the dye was not concentrated in the lumen. The tip of the tubule was then cut so that all the previous contents of the tubule were flushed out and only the distending fluid remained in the lumen. When the pressure was released and the walls collapsed the tubule began to concentrate the phenol red with its usual efficiency. It therefore appears likely that the active transport not only of water but of dissolved substances is adversely affected by distension.

DISCUSSION

Before proceeding further it is as well to be clear as to how far the evidence justifies the conclusion that water is actively transported. Most fresh-water animals produce urine which is hypotonic to the blood, and at first sight it would be natural to suppose that this involved the active transport of water. We know, however, that certainly in the Amphibia and probably also in most of the higher invertebrates there is a primary process of ultrafiltration followed by the reabsorption of salts, so that hypotonic urine can be produced without active transport of water. It could be argued that an ultrafiltrate was transported into the Malpighian tubule in one region and solutes reabsorbed in another. This argument cannot be refuted on the evidence so far obtained; one can but point out that there is no direct or circumstantial evidence in favour of the argument, and that since active transport of water is the simpler explanation the onus of disproof rests upon the opponents of this view.

It was an attractive feature of what might have been called the 'potassium theory' of urine formation that it accounted for the otherwise meaningless circulation of potassium as being necessary to maintain a current of water through the excretory system. This theory is now shown to be untenable. Water does not have to wait upon potassium but can be actively transported. It is true that the mechanism of

uric acid excretion proposed by Wigglesworth (1931) involves a circulation of potassium, but in fact the essential feature of the mechanism is the circulation of base, and Wigglesworth chose potassium rather than sodium to represent the base for no other reason than that the urate of potassium is the more soluble (personal communication). In any case it seems *prima facie* most unlikely that the amount of uric acid excreted bears any relationship to the very much greater amount of potassium circulated in the same time. The significance of the circulation of potassium remains obscure. It seems probable, however, that the movements of water are normally adjusted to the movements of solutes so as to produce urine which is more or less isotonic with the haemolymph. How this adjustment is brought about is not easy to see at present; the observed fact that urine production increases with dilution might have several explanations. It will presumably be necessary to study all the major constituents of the urine and the way in which their excretion is influenced by their concentrations in the haemolymph and in the urine before one can hope to see any clear picture, and it will be some time before this ground can be adequately covered.

SUMMARY

1. Single Malpighian tubules of the stick insect have been studied as preparations isolated in drops of haemolymph under liquid paraffin. Measurements of osmotic pressure have been made on haemolymph and urine.

2. The urine is more or less isotonic with the haemolymph over a wide (45-145%) range of osmotic pressure. But in a majority of cases the urine is slightly but significantly hypotonic to the haemolymph.

3. From these and other observations it is concluded that water must be actively transported across the wall of the tubule. The implications of this conclusion are discussed.

4. The normal rate of urine production is of the order of 0.07×10^{-3} cu.mm./sq.mm./min. The rate increases with increasing dilution of the haemolymph and vice versa.

5. Urine production normally ceases, probably as a result of distension of the tubule, when the pressure in the lumen rises to about 20 cm. H_2O .

I wish to thank Prof. Wigglesworth for his comments upon the typescript of this paper.

APPENDIX

After some consideration I have come to the view that the composition of the dissecting fluid used in these experiments should be put on record, although it has no special merits to recommend it. No complete analysis of stick insect haemolymph has been published. Figures for sodium and potassium have been given by Boné (1944), and more recently Duchâteau, Sarlet & Florkin (1952) have given a comprehensive list of the amino-acids and their concentrations. Based upon these observations and upon the general background of knowledge summarized by Wigglesworth (1953), a dissecting fluid was prepared as described below. Since micro-organisms very soon multiply in this fluid it was found

convenient to make it up fresh every few days from component solutions, each of which was stored in a number of sealed and sterilized bottles.

Component A (sugars)	Glucose	9 g./l.
	Sucrose	342 g./l.
Component B (amino-acids)	Glycine	1.25 g./l.
	Glutamic acid	2.8 g./l.
	Histidine	3.0 g./l.
	Lysine	1.6 g./l.
	Threonine	1.6 g./l.
	Valine	1.55 g./l.
Component C (alkaline earths)	CaCl ₂	2.7 g./l.
	MgCl ₂ ·6H ₂ O	8.1 g./l.
Component D (alkalis)	NaCl	11.9 g./l.
	NaOH	4.0 g./l.
	KCl	14.9 g./l.
	KOH	5.6 g./l.

Take 20 c.c. of A, 25 c.c. each of B and C, 5 c.c. of D. Make up to 100 c.c. Add more D drop by drop to adjust pH to 7.

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STUDIES ON THE MYONEURAL PHYSIOLOGY OF ECHINODERMATA

I. THE PHARYNGEAL RETRACTOR MUSCLE OF *CUCUMARIA*

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INTRODUCTION

Investigations of recent years have emphasized the differences between the myoneural systems of the skeletal muscles of the arthropods and the chordates. Myoneural facilitation determines the gradation of muscular contraction in the arthropods whose muscles are characterized anatomically by having relatively few motor axons; chordate muscles, on the other hand, show propagated muscle action currents, and gradation of response is effected by numerous motor axons.

In view of the possible relationships between echinoderms and chordates, it appeared desirable to extend our knowledge of the myoneural physiology of this group. Until recently information has been limited to two short notes by du Buy (1936*a, b*), describing experiments using the pharyngeal retractor of *Thyone* and a passing reference by Pantin (1935) to sea-urchin spines. While the present work was in progress a comparative study of the electrical responses of various smooth muscles, including those of *Thyone*, was published by Prosser, Curtis & Travis (1951).

MATERIAL AND METHODS

The mid-ventral pharyngeal retractor muscle of *Cucumaria sykion* (Lampert) was chosen for study. The muscle was obtained by making two longitudinal cuts on each side of the mid-ventral radius and continuing them anteriorly through the pharynx and the calcareous ring. Other pharyngeal tissue was dissected away, leaving the muscle attached to its pharyngeal ossicle and supplied by the radial nerve which lies along the under surface of the mid-ventral longitudinal muscle of the body wall (Fig. 1). The preparation was held down by a pronged pin placed over the ossicle. The free end of the muscle was attached to a light isometric lever. The whole preparation was immersed in sea water; the muscle was lightly loaded and allowed 3 hr. to relax. Most preparations show spontaneous contractions which occur every 20–60 min. and last for about 10 min.

A square-wave multivibrator which closely followed the design of Bernstein (1950) was used as a stimulator. The stimulating electrode was either a fine platinum wire or a silver-silver chloride fluid electrode. A fluid electrode with a fine slit aperture placed beneath the radial nerve was found to be more convenient for indirect stimulation. The anode was a square plate of silver foil.

Before commencing an experiment the sea water around the preparation was siphoned away and thereafter the preparation was periodically moistened. An interval of at least 4 min. was allowed to elapse between successive contractions to avoid fatiguing the muscle. In these conditions a preparation would remain active for at least 14 hr. Experiments were carried out at temperatures between 20 and 27° C. The temperature did not vary more than $\pm 0.5^{\circ}$ C. during any one experiment.

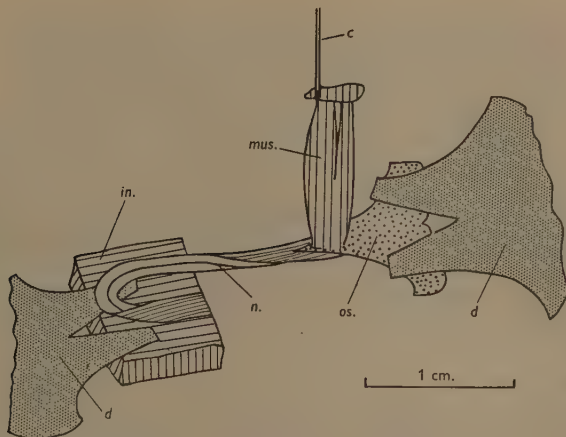


Fig. 1. Diagrammatic representation of the preparation used. The radial longitudinal muscle has been twisted to show the nerve *n.* *c.* attachment to the recording lever; *d.* pin; *in.* integument; *mus.* pharyngeal retractor muscle; *os.* calcareous ossicle.

HISTOLOGY OF THE PREPARATION

The retractor muscle has a single area of attachment to the calcareous ossicle, but divides distally into one anterior and two posterior fasciculi which may be further subdivided. The organization of the epimysium shows that this splitting is not due to damage to the muscle. Despite its fascicular architecture the muscle appears to function as a unit. The finer structure of the muscle agrees with that of the corresponding muscle of *Thyone briareus* Leseur as described by Olson (1938). There are in the muscle about 700 bundles of muscle fibres, each bundle containing two to fifteen fibres. Both the appearance of these muscle fibres and the organization of the fibre bundles is uniform throughout the tissue.

The muscle fibres are innervated by flat ribbon axons similar to those described by Smith (1950) in *Astropecten irregularis* (Pennant). The numerous cell bodies of the ribbon axons lie at the attachment of the retractor muscle to the ossicle and are connected by neurones within a motor complex to proximal motor neurone tracts of the radial nerve. The radial and circumoral nerves are very similar in structure to the corresponding nerves in *Marthasterias glacialis* (L.) as described by Smith (1937) and consist of longitudinally orientated neurones. It is estimated that a

section of radial nerve contains 600 axons, while at least thirty proximal motor neurone tracts run from the radial nerve to the motor complex. These neurones have a fibre diameter of 0.5μ and a length of several hundred micra. Histologically the preparation is similar to other known echinoderm nerve-muscle systems, the muscle being innervated by numerous motor axons. A detailed account will be published elsewhere.

RESULTS

Response of the muscle to direct stimulation

The muscle responds to a single direct stimulus with a smooth contraction irrespective of where the cathode is placed on the muscle. With a pulse length of 2 msec., the threshold of response is below 10 mV., the muscle responding with a minute contraction. As the intensity of stimulation is increased, the tension developed increases gradually to a maximal value. Fig. 2*a* shows a typical series of responses

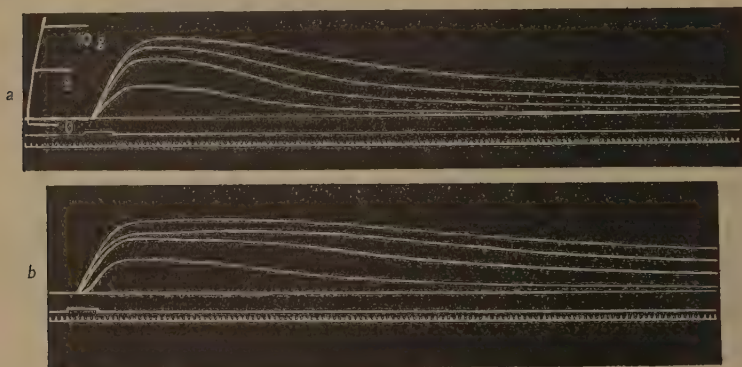


Fig. 2. (*a*) Response of the pharyngeal muscle of *Cucumaria* to direct stimulation at intensities of 6, 8, 9 and 10 V. (*b*) Response of the muscle to indirect stimulation at intensities of 8.0, 8.5, 9.0 and 10.0 V. Both recordings are from the same preparation. Stimulation frequency 5 stim./sec.; pulse length 2 msec.; time marker $\frac{1}{2}$ sec.

to stimuli of different intensities. If the intensity is held constant and the duration of the stimulating pulse varied, a similar relation, showing increase in tension with increasing pulse length, is found.

The response to different frequencies of stimulation was studied up to a maximal value of 400 stimuli/sec. (stim./sec.). Above fusion frequency the character of the response is unaffected by the frequency of stimulation (Fig. 3). Fusion occurs at about 2.5 stim./sec., and above 5 stim./sec. there is no further enhancement of tension.

Response of the muscle to stimulation of the radial nerve

That stimulation of the complex of radial longitudinal muscle and radial nerve results in the stimulation of the retractor muscle through the motor innervation

rather than by current spread, may be accepted from the following effects. First, the muscle fails to respond to stimulation of the radial tissues by a fluid electrode unless the aperture is placed accurately on the nerve. Again, if the nerve, which is easily ruptured by stretching, be broken, no response is obtained from the retractor muscle although the other tissues of the radius may be intact. Finally, both the nature of the response and the effect of stimulus frequency differ from those observed with direct stimulation of the muscle.

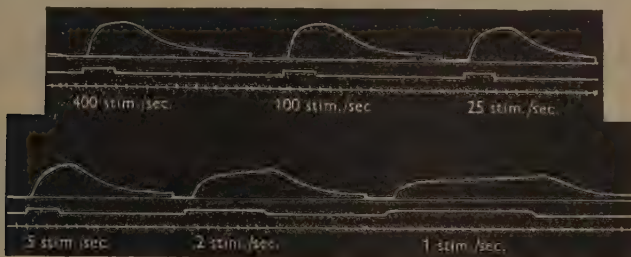


Fig. 3. Response of the pharyngeal muscle of *Cucumaria* to direct stimulation at an intensity of 23 V. and varying frequencies. Pulse length 2 msec.; time marker 1 sec.

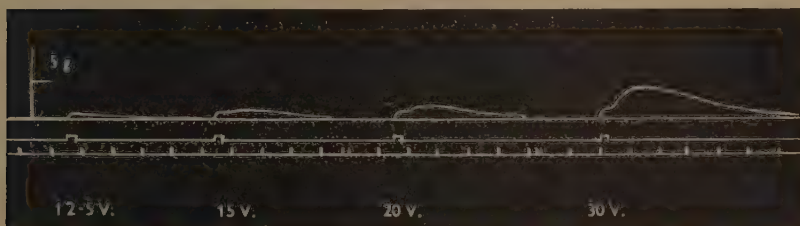


Fig. 4. Response of the pharyngeal muscle of *Cucumaria* to single shocks of increasing intensities applied to the radial nerve. Time marker 5 sec.

When the radial nerve is stimulated, single shocks are sufficient to elicit a response from the muscle (Fig. 4). This indirect response has a clearly defined double character with an initial fast contraction followed by a delayed one. The quick response attains maximal tension within 2 sec., while 5–13 sec. elapse before maximal tension is attained by the delayed response.

The threshold of the indirect response to single stimuli of 2 msec. duration varies from 2 to 10 V. This variation is probably largely due to differences in the thickness of the connective tissue layer overlying the nerve. For this reason it is not possible to compare values of stimulation intensity from one preparation to the next. The quick response has a lower threshold than the delayed (Fig. 4). The position of the stimulating electrodes determines the relative tensions developed by the two

responses. If a length of nerve greater than 0.5 cm. separates the cathode from the motor complex at the base of the muscle the delayed response develops the greater tension. With shorter lengths, so that the cathode lies just below the motor complex, the quick response predominates.

As the intensity of stimulation is increased, so the tension developed by the muscle increases to a maximal value (Fig. 2*b*). The general form of this relationship is to be seen in Fig. 5, and is independent of the frequency of stimulation. If the intensity is held constant and the pulse length varied instead, the tension developed increases with increasing pulse duration.

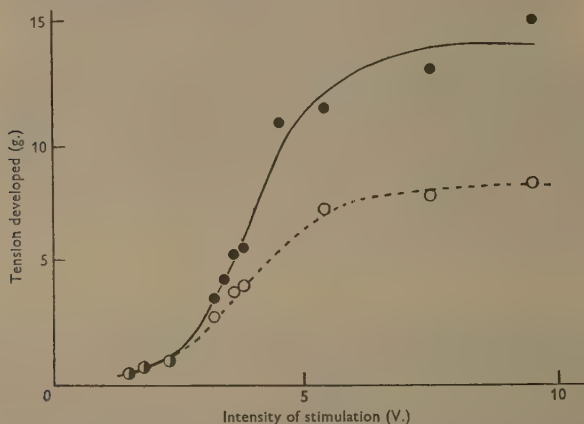


Fig. 5. Graph showing the relationship between the tension developed and the intensity of stimulation at a constant frequency of indirect stimulation of 10 stim./sec. The quick and delayed responses are shown separately. Full line, delayed response; broken line, quick response.

Low-frequency repetitive stimulation of 1.0–2.5 stim./sec. results in incompletely fused contractions. In the case of the quick response there is summation with each stimulus, but no marked increase in tension with increase in frequency of stimulation (Fig. 6). Above 2.5 stim./sec. the quick and the delayed responses fuse into a single smooth response (Fig. 2*b*), but the persistence of the delayed component in these conditions may be recognized by comparing the response to direct stimulation of the muscle (Fig. 2*a*) with that obtained by stimulation of the nerve (Fig. 2*b*). The longer duration of the maximal tension and the slower relaxation rate following indirect stimulation indicates that, at least at the three higher intensities, the delayed response has occurred. Above 10 stim./sec. there is a marked decrease in the tension developed and the two components of the response again become differentiated (Fig. 7).

The refractoriness which develops at stimulation frequencies above 10 stim./sec. is a character which distinguishes the indirect response from the direct (Fig. 8). It is shown by both quick and delayed responses as may be seen in Fig. 7. At high

frequencies the tension developed is no greater than that induced by a single shock. The extent to which this effect develops in the two responses at any frequency is a variable character. Examination of a number of preparations shows, however, no definite trend. The results are summarized in Fig. 9. For comparison all values

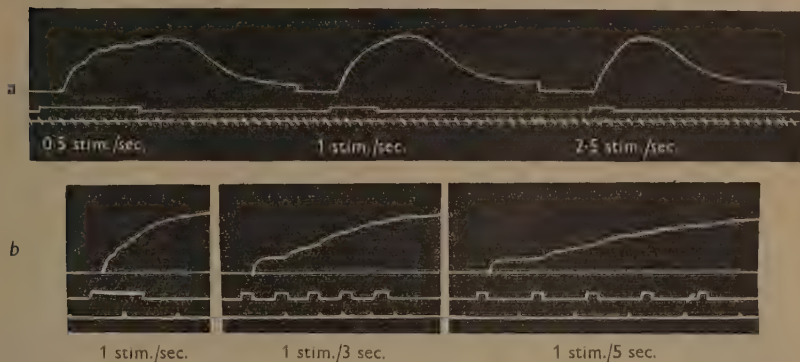


Fig. 6. (a) Response of the pharyngeal muscle of *Cucumaria* to five stimuli of varying frequencies applied to the radial nerve close to the motor complex. Intensity 2 V.; pulse length 6 msec.; time marker 1 sec. (b) Similar response at lower frequencies from a different preparation. Intensity 5 V.; pulse length 2 msec.; time marker 5 sec.

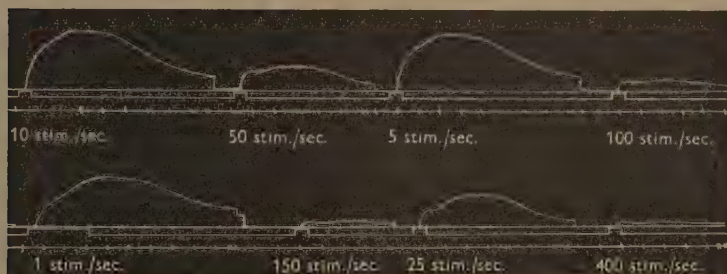


Fig. 7. Responses of the pharyngeal muscle of *Cucumaria* to varying frequencies applied to the radial nerve. The order of stimulation has been randomized to avoid fatigue effects. Intensity 4.2 V.; pulse length 2 msec.; time marker 5 sec.

have been reduced to a percentage of the maximal tension developed. Despite the considerable scatter, the results suggest that the extent of the development of refractoriness with frequency is similar for both responses.

It thus appears that for gradation of muscular response the complex here studied depends upon the recruitment of neurone paths, since tension development depends upon the intensity rather than the frequency of stimulation.

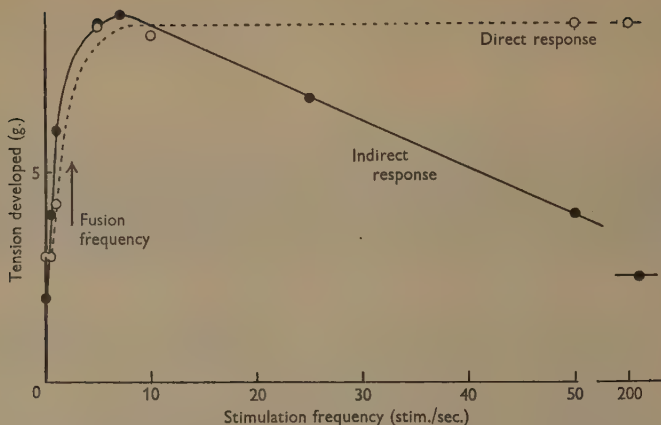


Fig. 8. Graph showing the relationship between the stimulation frequency and the tension developed, the intensity of stimulation remaining constant. Full curve, indirect stimulation; broken curve, direct stimulation.

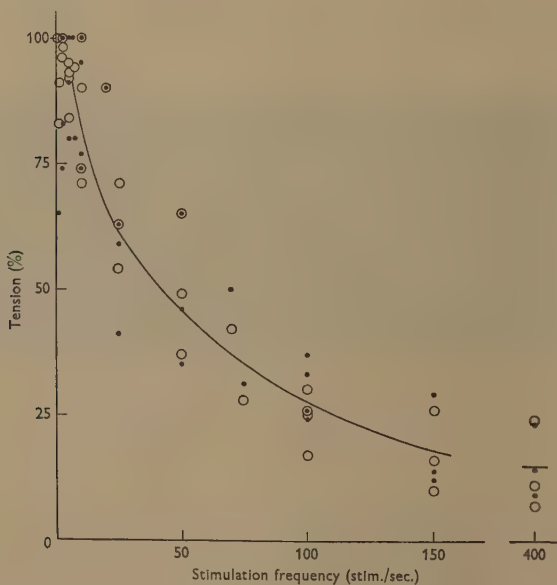


Fig. 9. Graph showing the relationship between the stimulation frequency and tension developed. The results are drawn from four different preparations and, for comparison, the tensions are expressed as a percentage of the maximal tension. Full circles, quick response; open circles, delayed response.

The quick and delayed responses

The origin of these two types of response must now be considered. It seems necessary first to decide whether the double response, which is not obtained when the muscle is directly stimulated, may not be an artifact. There appear to be four possible ways in which such an artifact might arise:

(i) The quick response might be the result of current spread from the point of stimulation. Since, however, destruction of the radial nerve by gentle pulling of the tissues destroys both responses, this cannot be the origin of the double response.

(ii) The delayed response may be a reflex arising from the initial rapid contraction of the muscle. As the delayed response does not appear when the muscle is made to contract by direct stimulation (Figs. 2*a*, 3), this interpretation can also be rejected.

(iii) The delayed response may be due to an after-discharge in the radial nerve. This seems very improbable, for the tension developed by the delayed response may be five to ten times as great as that developed by the quick response. Since, moreover, tension development is dependent upon the intensity and not upon the frequency of stimulation, this possibility can be excluded, as there seems no likelihood that more fibres are excited to after-discharge than are excited by the stimulating shock.

(iv) Finally, the delayed response cannot arise from the relay system around the circumoral nerve ring, as this is severed during the dissection of the preparation.

It has been pointed out above that the fast response has a lower threshold than the delayed (Fig. 4). The simplest explanation which can be offered of this fact is that there are two conducting paths along the radial nerve: one, having a low threshold, is responsible for the quick response; the other, with a higher threshold, produces the delayed contraction. The two responses have, moreover, very different latent periods, for examination of contractions recorded at high kymograph speeds shows that the delayed response cannot be extrapolated to the moment of origin of the fast response. A value of 0.90 ± 0.20 sec. has been found for the latent period of the quick response and 3.90 ± 0.18 sec. for the delayed. This difference in latent period might also be explained in terms of different conduction paths with different conduction velocities in the radial nerve.

To test this assumption of two distinct types of conduction path in the radial nerve, an attempt was made to determine the conduction velocities of the impulses associated with the two types of response. Using a knock-down key, the difference in time of onset of the contractions was determined when the radial nerve was stimulated at two different points a known distance apart. With this method there is no great difficulty in determining the conduction velocity of the impulses associated with the quick response. Twelve determinations, using five preparations, at temperatures of 24–27° C. were made. These gave a mean value of 0.17 ± 0.03 m./sec. In the case of the delayed response it is more difficult, by mechanical means, to obtain values of the conduction velocity owing to variations in the threshold along the length of the nerve. Only two satisfactory values were obtained. These were 0.28

and 0.17 m./sec. It is clear that in the radial nerve the conduction velocities of the impulses initiating both responses are of the same order, and that there is no possibility of associating the long latent period of the delayed response with low conduction velocity along the radial nerve. Nor does it seem likely that there are two distinct nervous paths, one responsible for the quick and the other for the delayed response.

It is known that along the motor paths supplying asteroid muscles lie complexes of motor neurones (Smith, 1950). Such a motor complex lies in *Cucumaria* between the radial nerve and the motor axons supplying the retractor muscle. The number of motor axons leaving the complex is far greater than the number of axons which supply it from the radial nerve.

It is clear that some distributory system must exist within the complex, and Smith has described an organization of internuncial neurones which are wholly intrinsic to the motor complex. The activity of this complex could be the origin of the delayed response.

In keeping with this suggestion is the fact that the rate of development of tension of the delayed response is less than that of the quick. Using single shocks, values of 3.1 ± 0.2 g./sec. were found for the quick response and 1.4 ± 0.03 g./sec. for the delayed. This difference may be attributed to a temporal dispersal of impulses arising from the motor complex. Such a dispersal could originate if the impulses from the input volley traversed different paths within the complex.

The lower threshold of the quick response necessitates the assumption that a certain liminal field of excitability must be reached by spatial summation to stimulate the internuncial neurones. This assumption is illustrated in highly diagrammatic and oversimplified form in Fig. 10. Neurones *A* and *B* arise from the radial nerve, and at the synapses *a* and *b* relay to motor nerves *A'* and *B'* running directly to the muscle and also at *c* to an internuncial neurone of the motor complex, supplying the motor nerves *C* and *D*. It is suggested that the internuncial neurone will only be excited when fibres *A* and *B* are simultaneously stimulated.

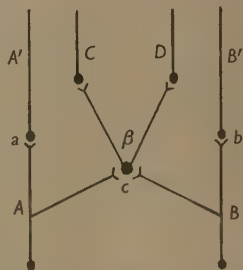


Fig. 10. Scheme of nervous connexions in the region of the motor complex. For explanation see text.

This picture requires that the axons of the proximal motor neurones coming to the motor complex and also the internuncial neurones of the motor complex should branch into a number of endings, and that the internuncial neurones should receive terminations from several fibres. Histological observations in favour of this view will be published elsewhere.

Finally, it is necessary to explain why, when the nerve is stimulated immediately beneath the motor complex, the quick response is stronger than the delayed, although the opposite is true when the nerve is stimulated more distally. It seems likely that the paths in the radial nerve running to the motor complex are augmented in the pharyngeal region by similar tracts from the sensory tentacles and possibly also

from the circumoral nerve. These paths would be stimulated by an electrode placed beneath the pharyngeal ossicle, but not by one more distally along the radial nerve. As a result the relative tensions developed by the quick and delayed responses vary. Immediately beneath the ossicle the quick response may equal the delayed, while with greater lengths of radial nerve the quick response may be only one-quarter that of the delayed.

Delayed facilitation

When two stimuli separated by several seconds are applied to the radial nerve, the delayed response to the second stimulus is greater than that to the first (Fig. 11 a). This effect usually lasts for about 60 sec., but may last as long as 120 sec. A typical

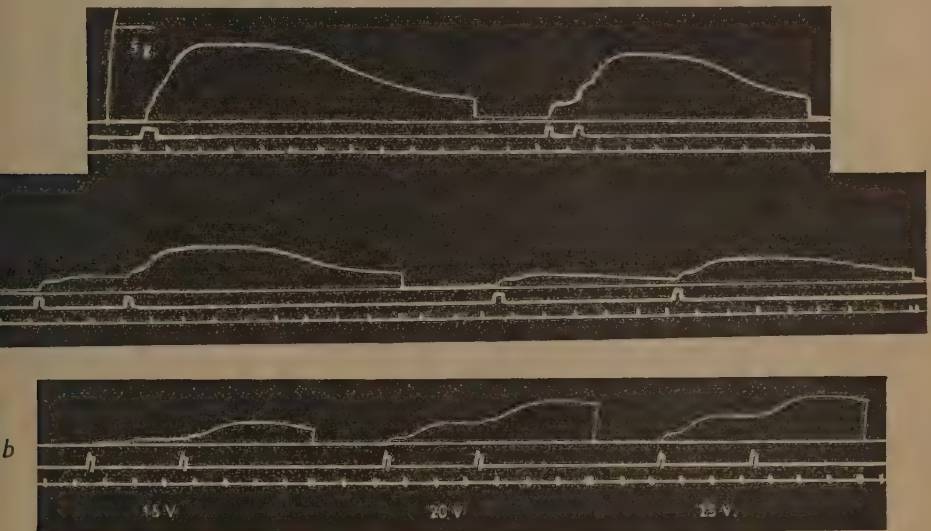


Fig. 11. (a) Responses of the pharyngeal muscle of *Cucumaria* to pairs of stimuli applied to the radial nerve. The intervals between the stimuli of each pair are 1, 5, 15 and 30 sec. Intensity 15 V.; pulse duration 2 msec.; time marker 5 sec. (b) Responses of the same muscle to a pair of stimuli at a time interval of 15 sec. and at intensities of 15, 20 and 25 V. Pulse length 2 msec.; time marker 5 sec.

set of values illustrating the decay of this facilitation with time is shown in Fig. 12. The facilitation is not found, in healthy preparations, with the quick response. Moreover, it does not occur when a preparation is stimulated at an intensity below the threshold of the delayed response: in these conditions there is neither facilitation of the quick response, nor does the delayed contraction appear in response to the second stimulus.

If the intensity of stimulation is raised so that the first response is almost maximal, the effect is not observed (Fig. 11 b). This suggests that the phenomenon is not due

to events at the myoneural junction. It seems more likely that this facilitation is due to more ribbon axons being excited by the second stimulus than by the first. The difference in this respect between the quick and the delayed response again suggests that the delayed response arises within the motor complex where the motor axons from the radial nerve supply several internuncial neurones. The effect may be compared to post-tetanic potentiation found in the vertebrate nerve cord (Lloyd, 1949) and in sympathetic ganglia (Larrabee & Bronk, 1947). In the sympathetic

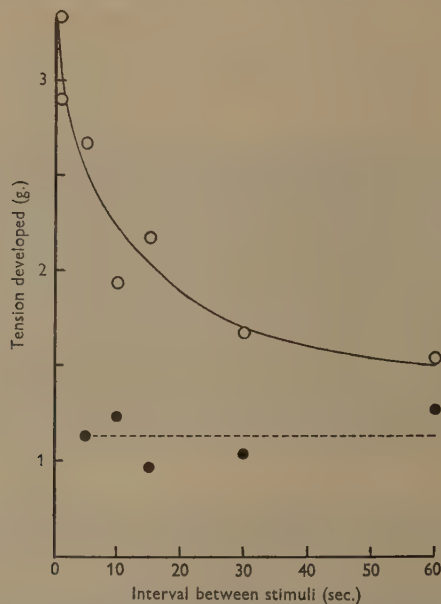


Fig. 12. Graph showing the relationship between the tensions developed by the delayed responses to pairs of stimuli of varying time intervals. Conditions of stimulation as in Fig. 11*a*. Full circles, response to first stimulus; open circles, response to the second stimulus of a pair.

ganglia of the cat the latter authors find that a single conditioning stimulus produces an initial facilitation which lasts about 0.1 sec. and a delayed facilitation which lasts for several seconds. It is to be expected that any such event will be more transitory in a mammal than in an invertebrate.

DISCUSSION

The original aim of this investigation was to decide whether there was to be found in an echinoderm evidence for myoneural facilitation such as has been demonstrated to occur in the coelenterates and arthropods. Pantin (1935) has previously suggested that such facilitation occurs in the muscles moving the spines of sea-urchins. The subsequent studies of Kinoshita (1941), have, however, shown that the organization of the peripheral nervous system is such as to make doubtful the

validity of Pantin's exploratory observations. With the quick response of the pharyngeal retractor of *Cucumaria* we have found no evidence which suggests that myoneural facilitation occurs. At low frequencies of stimulation the size of the steps of the staircase obtained is unaffected by the stimulation frequency (Fig. 6*b*), a condition which contrasts markedly with similar responses in *Calliactis* (Pantin, 1935). Moreover, there is almost no increase in the tension developed above fusion frequency. No evidence for delayed facilitation has been found with the quick response. With the delayed response it is not easy to affirm that no myoneural facilitation occurs, since the response appears at all frequencies of stimulation as a smooth contraction and the rapid onset of refractoriness prevents an effective study of the response over a wide range of frequencies. The delayed response shows marked delayed facilitation, but its absence when the radial nerve is maximally stimulated and its long duration of 60–120 sec. incline us to the view that this event probably occurs in the motor complex rather than at the myoneural junction.

In contrast to the coelenterates and arthropods, the system here studied is sensitive to the intensity of stimulation applied to the nerve. Such a result might indeed have been expected from a study of the histology of the preparation. In this property the preparation shows a similarity to a vertebrate nerve-muscle preparation. We have also suggested that spatial summation of excitation occurs in the motor complex as in the vertebrate nerve cord, and have shown the existence of a phenomenon similar to post-tetanic potentiation. It is tempting to see in these effects physiological evidence for a relationship between the chordates and echinoderms. However, the recent study by Hoyle (1952) shows that the responses of the siphons of ascidians, following electrical stimulation of the test, show many characters reminiscent of the responses of sea-anemones; in particular, there is no change in the magnitude of the response once the threshold of stimulation intensity has been passed, and there is evidence of the presence of a frequency-sensitive facilitation. It should, moreover, be remembered that although echinoderms and craniates have cholinergic nerves, no evidence for such a system has been found in the ascidians. Clearly if we accept the present results as favouring an echinoderm-chordate relationship, it becomes necessary to consider that the ascidians have evolved a specialized and distinct neuro-muscular system. In the absence of further evidence such speculation appears to be unprofitable.

Functionally the quick and delayed responses recall similar behaviour of the adductor muscles of lamellibranchs, and it seems likely that the quick response serves to bring about a rapid retraction of the pharyngeal ring, while the delayed response is the expression of a potential tonic mechanism for retaining the ring in a retracted position.

SUMMARY

1. A myoneural preparation of *Cucumaria sykion* (Lampert) comprising a pharyngeal muscle and the corresponding radial nerve is described. A brief account is given of the histology of the preparation.

2. Direct stimulation of the muscle gives a single response. Above fusion

frequency the tension developed by the muscle increases with increasing intensity of stimulation, but it is unaffected by the frequency of stimulation.

3. Stimulation of the radial nerve elicits a double response from the muscle. There is a 'quick' response which shows no myoneural facilitation and a 'delayed' response which shows a well-marked and prolonged facilitation.

4. The tensions developed by both quick and delayed responses depend upon the intensity of stimulation applied to the radial nerve. Both responses show well-marked refractoriness at stimulation frequencies above about 10 stim./sec.

5. It is suggested that the delayed response arises from the activity of the inter-nuncial neurones in the motor complex, and that this is also the locus of the prolonged facilitation shown by the delayed response.

6. The similarity of the properties of this system with certain features of the chordate nervous system are pointed out, but it is considered that any simple phylogenetic conclusions are unwarranted.

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AN ORGAN FOR PROPRIOCEPTION AND VIBRATION SENSE IN *CARCINUS MAENAS*

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(With Plate 6)

Little is known about the mechanism of proprioception in arthropods. A number of organs or groups of cells have been described which on anatomical grounds might serve proprioceptive function, e.g. the 'chordotonal organs' in insects (Graber, 1882) and crustaceans (Wetzel, 1933), bipolar cells in insect muscle (Hilton, 1924; Rogosina, 1928) or in crustacean joints (Tonner, 1933), the 'myochordotonal' organ in the meropodite of decapod crustaceans (Barth, 1934), the 'muscle receptor organ' and 'N-cells' of various crustaceans (Alexandrowicz, 1951, 1952*a, b*). Physiological evidence has been obtained for the crustacean 'muscle receptor organ' by Wiersma, Furshpan & Florey (1953), who showed that it gives rise to a discharge of afferent impulses during stretch, as was suggested by Alexandrowicz. The existence of kinaesthetic organs in the walking limbs of crustaceans was indicated by the fact that movements of the joints can elicit impulses in the limb nerves (Barnes, 1930, 1931) and in the central nervous system (Prosser, 1935). In insects there is evidence for a proprioceptive discharge from the campaniform sensilla (Pringle, 1938*a, b*, 1940) and the hair sensilla at the joints (Pringle, 1938*c*).

The present work arose from an interest in certain large nerve fibres (up to 20μ in diameter) in the limb nerves of *Carcinus maenas* which accompany the two large (30μ) motor axons to the flexor muscle of the dactylus. These were presumably sensory fibres. In preliminary experiments the nerves were traced to the periphery and found to end on an organ which lies across the propodite-dactylus joint. The response of the organ was then examined in isolation and *in situ*. The experiments, described below, indicate that it is concerned with proprioception and probably 'vibration-sense'.

METHODS

All experiments were performed on preparations from the walking legs of the shore crab, *C. maenas*. Except where stated, the histological and physiological descriptions apply to the organ lying in the propodite-dactylus joint.

Histological. The organs and nerves were stained with methylene blue, fixed in ammonium molybdate and mounted in xylene dammar, following the technique of Alexandrowicz (1951). In all cases, the organ and nerves were exposed in the leg and the entire limb immersed in methylene-blue solution. In some cases the preparations were counter-stained with picric acid.

Physiological. The preparations were dissected under a binocular microscope magnifying 20 diameters. Crustacean Ringer of the following composition was used

throughout: Na^+ , 5.13 mM.; K^+ , 12.9 mM.; Ca^{2+} , 11.8 mM.; Mg^{2+} , 23.6 mM.; Cl^- , 594 mM.; HCO_3^- , 2.6 mM.

In experiments on the isolated organ, the organ was tied at either end by a silk thread which was held by the tip of a ruling pen. The preparation was then placed in light liquid paraffin and the nerves laid on two recording electrodes, one of which was earthed. These electrodes consisted of chlorided silver wires dipping into agar-crustacean Ringer in capillary tubes and connected to the preparation by agar wicks. A cathode-follower, amplifier, oscilloscope and loudspeaker were used to pick up nerve impulses. The organ could be stretched or relaxed by altering the position of the ruling pens.

Its sensitivity to vibrations was tested as follows. A small glass rod, about 3 cm. long and 1 mm. wide, was mounted vertically on the diaphragm of a 'deaf-aid'. This was driven by a beat-frequency oscillator, the output of which could be varied over the range 0–20 a.c. V. and 0–16,000 cyc./sec. The earpiece was mounted over the organ so that the glass rod dipped into the liquid paraffin. The tip of the glass rod was adjusted to be about 1 mm. from the organ; its movement was never more than about 20μ . Since the oscillator output was low at low frequencies and the earpiece insensitive in this range, it was usually not possible to examine the effect of sinusoidal frequencies below about 100 cyc./sec.

In experiments on the isolated leg, the sensory nerves, the motor nerves to the flexor muscle of the dactylus and the motor nerves to the extensor muscle of the dactylus were separately dissected out in the meropodite. The entire preparation was then lifted into light liquid paraffin and the leg held by clamping the propodite. The sensory nerves were laid on two recording electrodes as in the isolated organ preparation; the motor nerves in turn were laid on two stimulating electrodes. These were of the same type as the recording electrodes and were connected through an isolating transformer to a stimulator from which pulses could be obtained at variable voltage, duration and frequency. Movement of the dactylus was registered by attaching it to an isotonic lever moving between a light source and a twin photo-electric cell. The differential response between the two cells caused by movement of the dactylus was led off to the second beam of the oscilloscope. A thread was also attached to the dactylus so that it could be moved in either direction from outside the preparation box. In this way the response of the organ to either active or passive flexion or extension could be examined. The dactylus could also be clamped in order to observe the effects of isometric contraction.

Conduction velocity in the nerves to the organ was determined by placing them (with the organ removed) in light liquid paraffin, stimulating maximally and recording at three or four points.

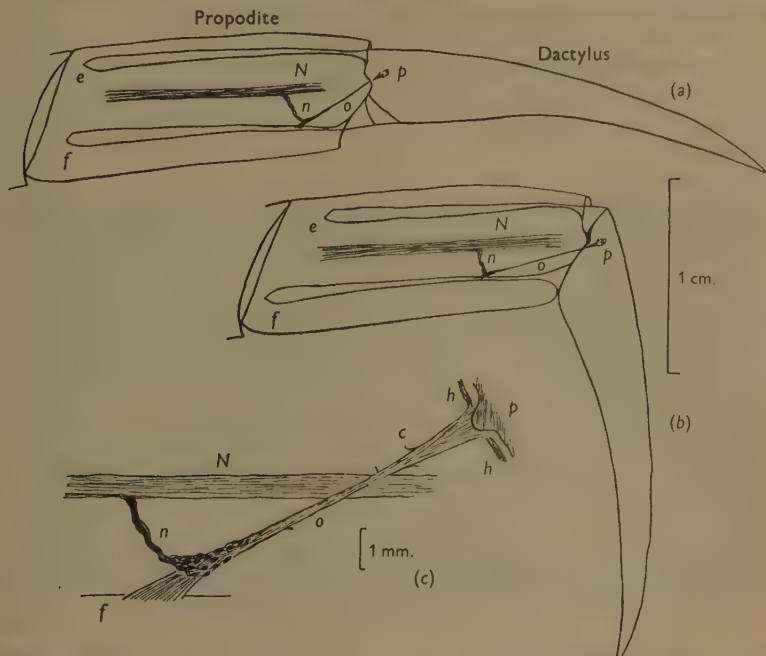
RESULTS

A. *Anatomy and histology*

The presence of a group of nerve cells in the organ spanning the propodite-dactylus joint was observed in unstained preparations, but their arrangement and connexions were shown only after staining with methylene blue. The staining also revealed

similar groups of cells in all three joints between ischiopodite and propodite, in each case on the flexor side.

The position and structure of the propodite-dactylus organ are sketched in Text-fig. 1. It consists of nerve cells and distal processes, embedded in a strand of connective tissue which runs across the joint. The strand is loosely attached to the



Text-fig. 1. Semi-diagrammatic sketches drawn to scale to show the position and structure of the organ. (a) Propodite-dactylus joint in extended position. (b) Joint in the flexed position. Note the elongation of the organ and the movement of the nerve (n). (c) Enlarged view of organ. *h*=hypodermis; *N*=main nerve bundle; *n*=nerve bundle to organ; *o*=organ; *p*=distal attachment to protuberance; *e*=apodeme of extensor muscle; *f*=apodeme of flexor muscle; *c*=small connective tissue attachment.

apodeme of the flexor muscle, about 5 mm. proximal to the joint; the other end is firmly attached to a small protuberance on the caudal inner surface of the dactylus, about 1 mm. distal to the joint. Some connective tissue fibres run to the hypodermis in the immediate vicinity of the protuberance, and a few small threads leave the organ along its length.

The organ is about 6 mm. long when the joint is extended. When the joint is flexed the organ is stretched by about 1 mm. due to the movement of the apodeme (see Text-fig. 1 *a, b*). It is about 100μ in diameter in the centre, widening at either end. The proximal processes of the nerves form a bundle which is free for about 2 mm. before reaching the main bundle, and is characteristically crinkled, presumably

to allow for the movement of the apodeme. The connective tissue strand is of very elastic material since it contracts, when its attachments are cut, to about half its length.

Pl. 6 shows photographs of a preparation stained with methylene blue. The large cells clustered at the proximal end of the organ are ovoid and up to about 40μ in diameter. The cells extend along the organ becoming smaller and more spindle-shaped and eventually impossible to distinguish from the 'beading' of the nerve fibres characteristic of methylene-blue staining. At least forty cells have been counted in one preparation and the number is probably greater because of uncertainties in staining. The lengths of the distal processes could not be accurately measured; some can be followed clearly for up to 200μ . Stained elements extend to the distal third of the organ.

The meropodite-carpopodite organ lies across the joint and resembles the propodite-dactylus organ in its attachments and general appearance but is smaller in size.

The only other species examined was *Portunus depurator* in which the propodite-dactylus organ is present and resembles that of *Carcinus*.

The groups of bipolar cells described by Tonner (1933) are similar to those described here, but apparently not associated with a muscle. The organ seems related to the 'myochordotonal' organ of Barth (1934) in its general structure, large number of cells and association with a muscle. The points of difference are that the 'myochordotonal' organ does not cross a joint, has smaller cells (up to 10μ in diameter) and makes contact with the muscle only through a membrane in which the cells lie.

B. *The discharge of the isolated organ*

A resting discharge which might continue for several hours was recorded from the nerves and this discharge could be modified in a consistent manner by certain mechanical stimuli. If the nerves were severed or crushed near the organ, both resting discharge and response to sensory stimulation ceased. Crushing the organ from the distal end progressively reduced the discharge. The discharge was also reduced or abolished by a drop of 0.2% procaine placed on the organ. It may be inferred that the discharge originates in the organ and is sensory in nature.

Only very small potentials or none at all were recorded from the organ itself; no prolonged depolarization of the type found near the nerve terminations of a muscle spindle (Katz, 1950*a, b*) was seen. It is probable that the nerve within the organ is sufficiently long for the action potential to have reached its normal shape at the point of nerve exit. Any local electrical events inside the organ are presumably short-circuited and, therefore, not recordable.

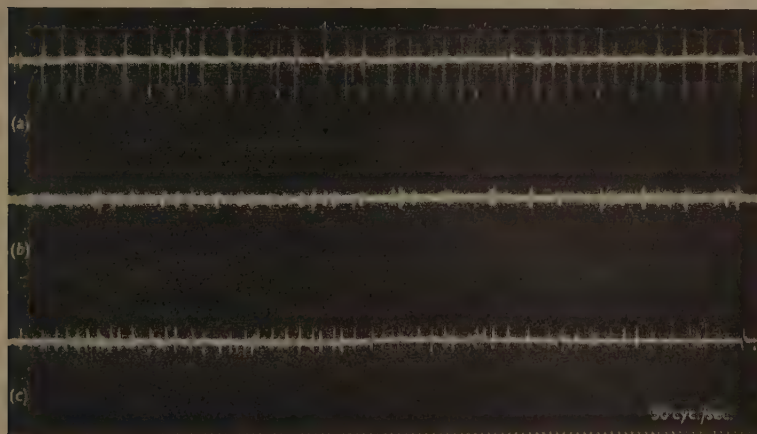
The resting discharge varies with the length of the organ (see Text-fig. 2). When the organ is allowed to shorten as far as it can, the discharge becomes greater than it is at the lengths normally attained in the leg. In the physiological range of lengths, the rate of discharge in some fibres is dependent, in others apparently independent of length.

Judging from the size of the spikes the resting discharge appears to occur in small

fibres. When the organ is suddenly stretched or quickly allowed to relax, there is a burst of large spikes more or less coincident with the movement. These impulses travel evidently in large fibres which are normally at rest. Rapid stretch is usually more effective in eliciting this discharge than is rapid relaxation.

Vibratory stimuli

The sensitivity of the isolated organ to vibration was very high. The organ responded to a tap on the bench or even to heavy footsteps several yards away. The freedom of movement of the organ in the isolated condition was much greater than in the leg, yet it would respond to a vibration which caused no movement visible to the naked eye.



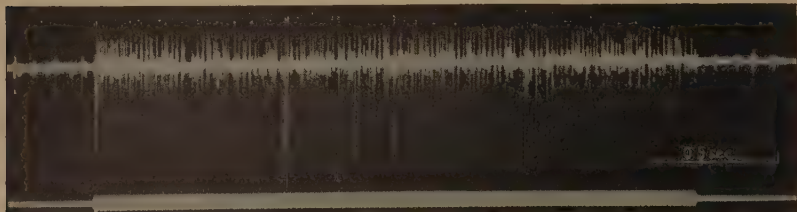
Text-fig. 2. Resting discharge in the isolated organ and nerve. (a) Organ at length of 2.7 mm. (the length taken up when attachments were cut.) (b) Organ at length of 4.5 mm. (the length in the extended position of the joint). (c) Organ at length of 5.9 mm. (the length in the flexed position of the joint). Note that different fibres may come in at different lengths (compare (a) with (b) and (c)) and that the same fibres may show different frequencies (compare (b) with (c)).

Many axons, but especially the large fibres which respond to sudden stretch or relaxation, can be caused to discharge during vibration. When the organ is stimulated with the 'vibrator' (see Methods) the type of record shown in Text-fig. 3 is obtained. There was no indication that any unit was tuned to a particular frequency within the band employed. The higher the frequency the higher was the intensity of stimulus required to produce the same response. With the present apparatus no response could be obtained above 1000 cyc./sec.

The rate of firing in any one unit during a vibratory stimulus was irregular and did not follow the frequency of the stimulus. The maximum mean rate of firing observed in a single unit over a period of 200 msec or more was 100 impulses/sec. The minimum interval seen between two impulses was 4 msec. (temperature

17.5–20° C.). The response to continuous vibration showed little or no adaptation (see Text-fig. 3).

In one experiment, the meropodite-carpopodite organ and nerve were mounted in liquid paraffin. Resting discharge and response to a brief vibration were similar to those of the propodite-dactylus organ.



Text-fig. 3. Response of the isolated organ to vibrational stimulus at 350 cyc./sec. Signal on lower beam indicates the duration of the stimulus. The large spikes have been retouched.

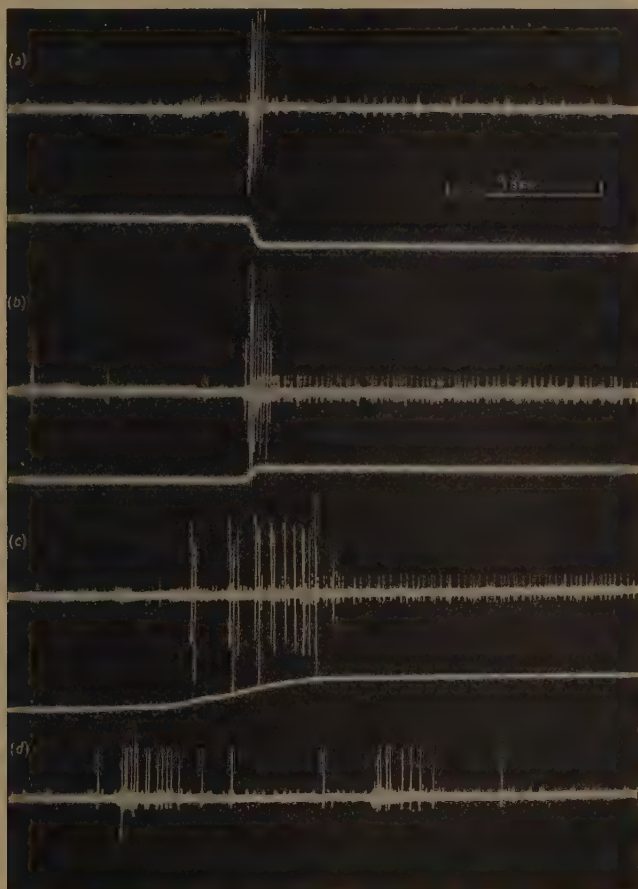
C. *The discharge of the organ in situ*

A recording could be made from the nerve bundle to the organ by exposing it in the meropodite (see Methods). This bundle is easily recognized by its large fibres and is fairly discrete. Its identity was always confirmed after the experiment by dissecting it up to the organ. In some cases, a few nerve fibres from other bundles were present, but in these experiments the results differed in no way from those in which only the bundle supplying the organ was used.

The resting discharge occurred in small fibres and was similar to that in the isolated preparation, although it disappeared sooner, possibly due to asphyxia and accumulation of metabolites from contracting muscle. The discharge varied with the position of the joint, certain units discharging at different rates according to the position, others behaving in an irregular way. The units which respond to positional changes adapt slowly if at all, the result being somewhat obscured by the gradual deterioration mentioned above.

The effects of passive movements and vibration are shown in Text-fig. 4 and of active movements in Text-fig. 5. With the exception of the effects due to a prolonged stimulus to the motor nerves there are no obvious differences between them. The active movements were produced by applying a series of stimuli to the motor nerves, the frequency being varied according to the speed of movement required. The duration of stimulation was $\frac{1}{4}$ – $\frac{1}{2}$ sec. for a brief contraction. For a prolonged contraction, stimulation was maintained for 4 sec. or more. The possibility that electrical stimulation of the motor nerves might accidentally stimulate the sensory nerves was ruled out: (i) by crushing the sensory nerves between the recording electrodes and the organ, thereby abolishing all discharge; (ii) by the fact that if the movement was delayed the sensory discharge was likewise delayed and coincided with it; (iii) because the difference between isotonic and isometric contraction of the flexor muscle (see below) can only be explained by active movement.

The results are summarized in Table 1. The phasic discharge takes place primarily in the large fibres and depends on the rate and extent of movement. The faster the movement the greater the *frequency* of discharge (see Text-fig. 4*b, c*). The



Text-fig. 4. Response of the organ to vibration and passive movement of the propodite-dactylus joint. Recording from the nerve in the meropodite. The lower beam indicates movement of the dactylus. (a) Passive extension; (b), (c), passive flexion at different rates; (d) two taps on preparation box (no signal): (a), (b) and (c) from the same experiment. Large spikes retouched.

farther the joint is moved the greater the *number* of impulses at a given rate (see Text-fig. 4*c*). It is possible to reduce the discharge considerably, and perhaps avoid it altogether, by moving the joint very slowly. Flexion is more effective than extension in eliciting a discharge.

When the leg relaxes, after brief muscular contraction, there is usually a second discharge similar to that during passive stretch and relaxation (see Text-fig. 5 *a, c*). The effect of a maintained contraction, however, differs from that due to a passive change of position. With prolonged contraction there is a continuous sensory discharge, often in large fibres, in spite of the fact that visible movement has ceased (Text-fig. 5 *b, d*). The explanation may be that the contraction is really an incompletely fused tetanus, the irregularities of movement being too small to be recorded but sufficient to stimulate the receptors.

Table 1. *Static and phasic responses from the organ*

(—, means absence of discharge; +, firing rates roughly indicated.)

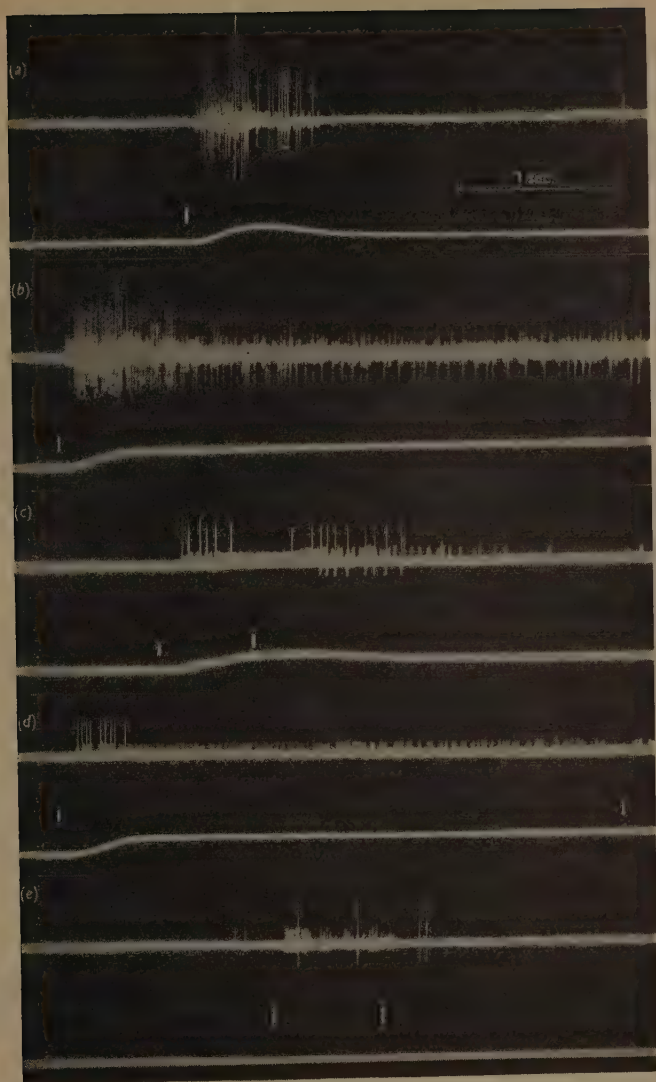
	Large fibres	Small fibres
Position (flexed)	—	+
Position (extended)	—	+
Movement (active or passive flexion)	+++	+++
Movement (active or passive extension)	++	+++
'Isometric' contraction (flexor muscle)	+	++
'Isometric' contraction (extensor muscle)	—	+
Vibration	+	++

Isometric contraction of the flexor muscle produced a similar though less intense sensory discharge, in spite of the fact that no external movement was allowed (the dactylus being clamped) (Text-fig. 5 *e*). This result is not unexpected since contraction will move the apodeme slightly. Isometric contraction of the extensor muscle produced no discharge, which is probably explained by the fact that this muscle has no direct connexion with the organ.

A brief vibrational stimulus—a tap on the preparation box—produced a discharge in both large and small fibres (see Text-fig. 4 *d*), the intensity of the discharge increasing with the strength of the stimulus. The sensitivity was much lower than with the isolated preparation, but the vibration was still effective without causing any recordable movement of the joint. In view of this, it is not surprising that an *apparently* smooth tetanus gave rise to continued discharges in the vibration receptive axons.

Conduction velocity in the nerves

The fastest speed of conduction measured was about 3.5 m./sec. at 21.5° C. There are perhaps a dozen fibres with velocities above 1 m./sec. and many whose velocities could not be measured. Because of the large number of nerve fibres in the bundle, the conduction of individual impulses may be regarded as taking place in a medium of low resistance. The fastest fibres are thus comparable to the motor axons whose conduction velocities in sea water range from 3.1 to 5.5 m./sec. at 21° C. (Hodgkin, 1939).



Text-fig. 5. Response of the organ to contraction of the flexor and extensor muscles of the dactylus produced by stimulation of the motor nerves. The beginning and end (where visible on the original photograph) of the series of stimulus artifacts are indicated by white bars. The second beam records movement of the dactylus but, because of an adjustment to the lever between (b) and (c), extensor of the joint in (d) and (e) is shown in the same direction as flexion in (a) and (b). Recording from the nerve in the meropodite. All records from the same experiment. Large spikes retouched. (a) Active flexion (brief contraction). (b) Active flexion (maintained contraction; end of stimulus off the photograph). (c) Active extension (brief contraction). (d) Active extension (maintained contraction). (e) Brief 'isometric' contraction of flexor muscle.

DISCUSSION

The organ can signal the rate and extent of a movement of the joint. Whether it can signal the direction of the movement or the resting position of the joint is not clear from the experiments, although there are some indications that this might be done by a variation in the pattern of the discharge in different fibres. Judging from a single experiment, these conclusions can probably be applied to the meropodite-carpopodite organ.

The fact that movements are signalled by large fibres whose conduction velocity approaches that of the motor axons is probably important in eliciting rapid reflex action.

The arrangement of the organ seems to provide better shielding against extraneous stimuli and give a more accurate 'muscle-sense' than, for example, the campaniform sensilla or the hair sensilla in the joints of insects. Unlike the 'muscle receptor organ' in crustaceans (Alexandrowicz, 1951) and the vertebrate muscle spindle, the present crab-limb organ lies in series with the main muscle bundle; contraction of this muscle therefore stretches the crab-limb organ while it relieves the tension on the other two. The crab-limb organ also differs from the others in that it responds to sudden relaxation as well as sudden stretch. This suggests that the movement receptors undergo only transient deformation such as might be produced by friction or viscous drag during the movement.

It is more difficult to decide whether the crab uses the organ to perceive vibrations. Many tissues which cannot be regarded as specific 'vibration-receptors' respond incidentally to a vibrational stimulus (e.g. the teeth, Pfaffmann, 1939; the skin, Adrian, Cattell & Hoagland, 1931, Newman, Doupe & Wilkins, 1939). Any receptor which is excited by mechanical deformation will probably respond to vibration of sufficient intensity; the question is whether normally the tissue experiences vibrations of greater than threshold intensity. The stimuli used on the isolated leg of the crab were small, and it is reasonable to suggest that the crab can perceive vibrations due to nearby moving objects. It might seem unusual that the same fibres should convey two different types of mechanical sensation. However, there is good reason to believe that in vertebrates the same end-organs (stretch receptors in the muscle) are responsible both for deep vibratory sensibility and for appreciation of changes in posture (Echlin & Fessard, 1938; cf. also Newman *et al.* 1939). The suggestion of Echlin & Fessard (1938) that the sensations can be resolved centrally because of the different pattern of the two types of discharge seems plausible.

The present experiments may also explain the isolated-leg 'reflexes' described by Barnes (1931). Barnes found, for instance, that passive flexion of the dactylus of the isolated limb of *Eupanopeus* produced a maintained active flexion. Supposing that the nervous anatomy is similar to that in *Carcinus*, the initial flexion will produce a discharge of impulses in nerves which lie in close proximity to the motor nerves to the flexor muscle. 'Cross-excitation' of these motor nerves might take place near the cut ends.

SUMMARY

1. An organ lying across the propodite-dactylus joint of the walking leg of *Carcinus maenas* has been described; this organ is supplied by a nerve bundle which contains some large fibres (up to 20μ in diameter) and which accompanies the motor nerves to the flexor of the dactylus. A similar organ lies in the meropodite-carpopodite joint.

2. The organ is embedded in a strand of elastic tissue which is in a stretched condition at all positions of the joint, the stretch being greater the more the joint is flexed.

3. The organ with its afferent nerve has been isolated and shown to contain sensory receptors. It reacts to vibration and to sudden changes in length with a burst of impulses in both large and small fibres. There is a resting discharge in small fibres which varies with length.

4. Experiments on the organ *in situ* show that it can serve to signal the rate and extent of movement in the propodite-dactylus joint. In addition, it may be used as a vibration-receptor. It is probable that some of its nerve fibres can signal the position of the joint and the direction of movement.

Some preliminary experiments were done with Dr Xenia Machne to whom I am greatly indebted. I am extremely grateful to Dr J. S. Alexandrowicz for showing me his methylene-blue technique and for much helpful advice. My thanks are due to Prof. B. Katz for constant encouragement and advice, to Mr J. Armstrong for the photographs of the organ, to Miss A. Paintin and Mr K. Copeland for technical assistance, and to Mr J. L. Parkinson for constant help.

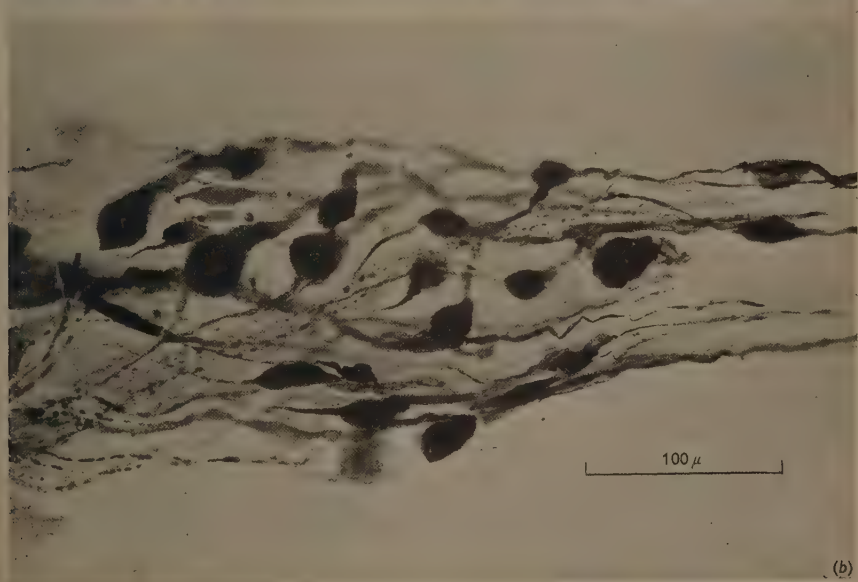
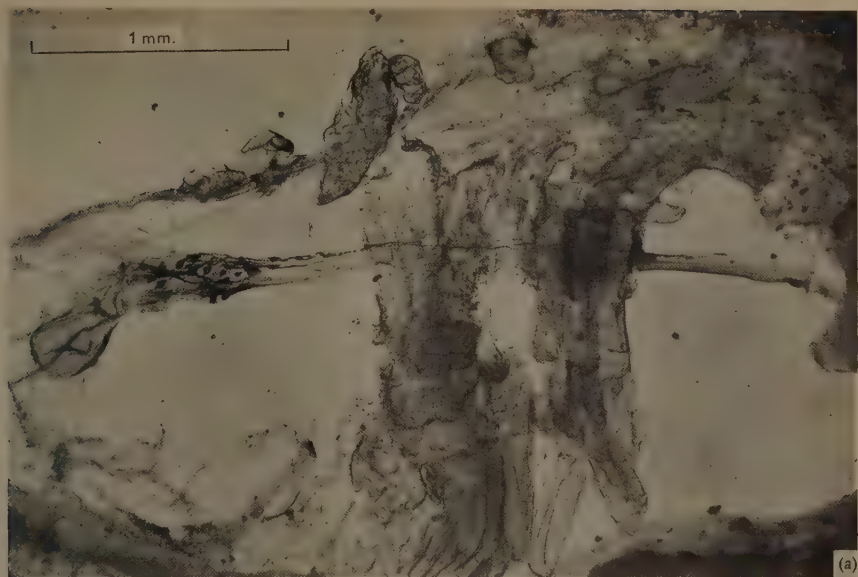
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EXPLANATION OF PLATE 6

Propodite-dactylus organ and nerve supply. Photo-micrographs made from a preparation stained with methylene blue, fixed with ammonium molybdate and mounted in xylene dammar. (a) Low-power photograph showing almost the entire organ lying across the centre, the nerve supply (two branches) joining at the left. The proximal attachment of the organ is seen faintly on the left, the distal attachment is outside the picture to the right. The cells and nerve processes can be seen extending along the upper part of the organ. Other material in the photograph is flexor muscle and hypodermis. The organ has been damaged slightly in the distal region and has been allowed to shorten by about a third in fixation. (b) High-power photograph of the nerve-organ junction in the same preparation. Distal part of the organ on the right.



BURKE—AN ORGAN FOR PROPRIOCEPTION AND VIBRATION SENSE
IN *CARCINUS MAENAS*

INTERNAL HYDROSTATIC PRESSURE OF THE *FUNDULUS* EGG

I. THE ACTIVATED EGG*

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I. INTRODUCTION

The egg of the killer minnow, *Fundulus*, has presented the physiologist with an exceptional problem because of the claim still extant that it is impermeable to water. J. Loeb found that the fertilized *Fundulus* egg could develop equally well in sea water of varying tonicities and even in tap water, as well as in distilled water. He noticed (1912) that when placed in hypertonic solutions of balanced mixtures of salts, the whole eggs remained floating and continued developing for 10 days, whereas newly hatched larvae died in a few minutes. These results indicated to him that the egg did not lose or gain either salt or water to affect the density or development of the ovum. When, however, the eggs were transferred to hypertonic solutions of single or unbalanced combinations of salts, they sank within a short while. This was to him a sign of permeability to water as an indication of damage. The apparent impermeability to water and electrolytes was attributed by Loeb exclusively to the tough chorion surrounding the egg, which became permeable only when injured by unbalanced salt solutions. Death of newly hatched larvae in hypertonic media was considered as confirmatory of the protection given by the chorion.

Later works of Bodine (1928) and Armstrong (1928), working with whole eggs as well as eggs with the chorion surgically removed, indicated that the presence or absence of the chorion did not materially alter the lethal action of unbalanced salt solutions on the egg. Yagle (1930), using whole and dechorionated eggs, confirmed Loeb's flotation experiments and came to the conclusion that not only the chorion was impermeable to water and electrolytes but also the vitelline membrane on the surface of the egg. In any event, the *Fundulus* egg has been considered as unique because of the apparent impermeability to water and electrolytes during its healthy state (Krogh, 1939; Brooks & Brooks, 1941). Indeed, this condition has been spoken of as a regulatory mechanism by which the *Fundulus* egg maintains its osmotic concentration in environments of various tonicities from that of sea water to that of brackish water (Prosser, 1951).

A counterpart of the marine *Fundulus* egg has been suggested to be the egg of the fresh-water trout. Gray (1932) claimed that the healthy trout egg was completely

* Supported in part by a grant from the American Philosophical Society.

impermeable to water and electrolytes. Impermeability to water was indicated by the absence of any appreciable change in the weight of the egg transferred to Ringer's solutions of greatly hypertonic strengths. On the other hand, impermeability to electrolytes, i.e. lack of escape of electrolytes, was indicated by no increase in conductivity of the distilled water in which healthy trout eggs had been immersed. Svetlov (1929) by freezing-point determinations of the various components of the trout egg had claimed that the chorion of the trout egg was freely permeable to both water and electrolytes. He found that by changing the composition of the external medium, the freezing-point of the whole egg (egg proper and perivitelline fluid) altered by an amount which could be accounted for by the replacement of the perivitelline fluid by the new external medium. Gray accepted Svetlov's conclusions, but claimed the impermeability to be due to 'the static properties of the vitelline membrane which excluded the passage of water and electrolytes' from the egg proper. This impermeability he claimed to be responsible for the maintenance of the relatively high osmotic concentration within the egg (f.p. = -0.48° C.) as compared to its native fresh-water environment (f.p. = -0.02° C.). Similar to the *Fundulus* egg, the trout egg was supposed to develop permeability to water and electrolytes only when it was injured, either mechanically or through ageing.

Except for the work of Krogh & Ussing who found no evidence for the penetration of heavy water (1937), the investigations indicating lack of water permeability of the *Fundulus* egg and of the trout egg have depended chiefly on changes in weight or density of the eggs placed in different environments.* It was assumed that if the eggs were permeable to water, then their weights in hypertonic media would decrease and their densities increase because of loss of water to the external environment. This was the basis of Loeb's flotation experiments and Gray's observation of the trout egg proper floating within its own chorion in hypertonic media. These observations indicated no net changes in weight and density, but cannot indicate transitory changes at any specific time, which is needed to appreciate progressive changes with time. In view of the importance of the conclusion that these two species of eggs are completely impermeable to water, the problem was reinvestigated on the *Fundulus* egg with different techniques designed to show transitory changes as well as net alterations in water exchange. The method employed consists of simultaneous determinations of pressure and volume of the egg during early development and under different experimental conditions. Whenever movement of water occurs, there is either a volume change or a pressure change or more likely a combination of the two. In sea-urchin eggs permeability to various substances has been studied by means of volume changes (Lucke & McCutcheon 1932). Information would be more complete if it were possible to combine a study of both pressure and volume changes. The results of this and succeeding papers on the *Fundulus* egg present evidence indicating free permeability of the egg to water.

* The extensive work on the action of acids and various salt solutions on the *Fundulus* embryo is not discussed in our papers which are concerned solely with water permeability. A great difference also exists between the stages of development. In our work only freshly stripped eggs were used, and then only in the first few hours.

This has been accomplished by means of simultaneous pressure and volume studies. In the present paper is described the method of study, and the internal hydrostatic pressure of the unfertilized *Fundulus* egg upon artificial activation and during its subsequent development in sea water.

II. METHOD

The apparatus used for recording pressure was a mercury manometer. As shown in Fig. 1, there were two outlets to the manometer: one of these was connected to a micropipette, and the other to a syringe with a screw adjustment. The lower part

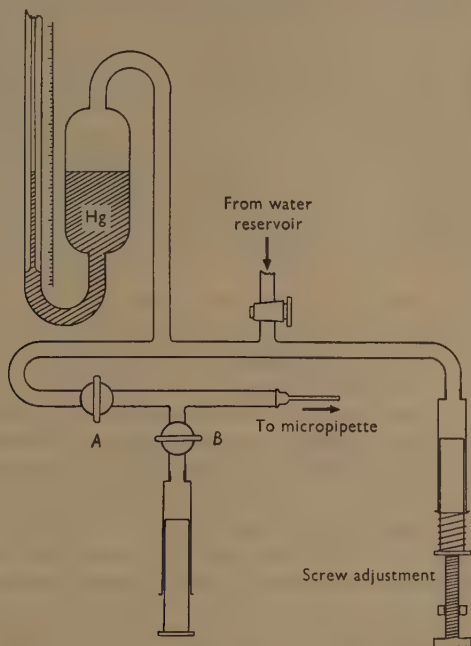


Fig. 1. Schematic diagram of the manometer used in the experiments. Except for the portion occupied by mercury, the entire system is filled with water. The side syringe is connected through valve *B*. When the micropipette has been inserted into an egg, valve *B* is closed and valve *A* opened. Pressure changes are effected by the screw adjustment.

of the bulb and the lower U-bend were filled with mercury, while the rest was filled with water, including the entire length of the micropipette except for the tip of the shaftlet which was filled with a column of oil. In this manner a complete hydraulic system was accomplished, and a pressure exerted anywhere in the system would be transmitted equally to all points. A side syringe was provided to facilitate various adjustments, such as drawing up of the oil into the tip of the micropipette, assisting in the adjustment of zero-pressure level, microinjection, etc.

The technique of operation in detail is as follows: Convenient lengths of capillary tubing, 0.8 mm. outside diameter, were washed, dried and one end drawn over a microflame, into a suitable micropipette having a gradually sloping, conical shaftlet terminating in a closed tapering tip. The coarser, open end of the micropipette at the opposite end of the microtip was then sealed in a flame and several such pipettes set aside for use. When ready for operation, the sealed, coarser end of the micropipette was broken open and dipped into a drop of thin paraffin oil coloured orange with Sudan III. A very short length of the oil entered the open end of the lumen by capillarity, while the greater part of the lumen of the micropipette, extending to its tapering tip, remained filled with air. The main shaft of the micropipette, with the column of oil at the open end, was then coupled to a standard, water-filled, metal pipette holder of the micromanipulator, the holder being connected to the hydraulic system of the apparatus. No air was allowed to interpose between the water in the metal holder and the oil in the coarse end of the micropipette. After a tight connexion had been achieved, a slight pressure was exerted on the side syringe, driving the water, with the oil ahead of it, along the shaft of the micropipette and towards the microtip. Meanwhile the tip of the micropipette was carefully broken to the desired diameter of $5\text{--}10\mu$ while under observation through the microscope. Further pressure displaced water in the shaft with the oil column in advance of it towards the microtip. When the oil reached the tip and the entire micropipette was filled with fluid, the hydraulic system was ready for use. Valve *B* (Fig. 1) of the side arm was then closed and valve *A* opened, thereby connecting the micropipette with the mercury manometer.

At the beginning of each experiment, the mercury column was adjusted to zero reading through the screw adjustment, with the tip of the micropipette just beneath the surface of the sea water in the dish containing the eggs to be operated upon. The pipette was then inserted into an egg which would displace a small amount of its material into the micropipette provided there was an appropriately lower pressure in the pipette. When this occurred, a meniscus was created between the oil in the pipette and the fluid exuding from the egg. By moving the syringe to manipulate pressure this meniscus was maintained close to the inner surface of the chorion of the egg. Under these conditions the egg was thus at the nearest physiologic state of pressure found practicable. If there were any increase or decrease of pressure in the egg, and if the meniscus were carefully maintained at this level by turning the screw adjustment, all pressure changes so caused were transmitted to the mercury column. The height of the column in the mercury manometer represented the pressure in the egg at any given time.

Eggs were stripped from a female into a dish of sea water. The bottom of the dish had been covered with a layer of paraffin wax into which were cut several depressions, slightly larger than a *Fundulus* egg. These depressions serve to hold the eggs while being punctured under microscopic observation. Punctures were made with the tapering tips of diagonally directed micropipettes having a bore of $15\text{--}20\mu$ for a distance of $\frac{1}{2}$ mm. from the tip which averaged $5\text{--}10\mu$ across. These sizes were found to be most convenient for recording pressures, since with narrower pipettes, the movement of the meniscus became sluggish, making it difficult to observe rapid changes in pressure.

In the experiments being reported, measurements were made of (1) the time at

intervals beginning from the moment of puncture, (2) internal hydrostatic pressure of the egg, (3) the outside diameter of the chorion, and (4) the diameter of the egg proper.

III. OBSERVATIONS AND RESULTS

A. *Morphology of the Fundulus egg and effect of puncture*

The egg of the *Fundulus* found in Woods Hole waters is spherical, fairly constant in size from the same batch, and averages 1.8 mm. in diameter. It possesses an outer relatively thick coat, the chorion (c. 10μ), which is flaccid during the first few minutes after it has been stripped into sea water. From then on, the chorion is inelastic and maintains a constant diameter. The eggs rapidly become turgid. Closely applied to the under-surface of the chorion lies the egg proper which consists chiefly of yolk material and several relatively large oil globules. The protoplasmic portion of the egg is spread out in a thin layer over its periphery. Just below the surface of the egg proper is a layer of closely packed, small, colourless spherules, the so-called platelets. When the unfertilized egg is left standing in sea water, it becomes spontaneously activated, undergoing a sequence of events similar to that occurring after insemination (Kagan, 1935). These consist of disappearance of the platelets, development of a perivitelline space, and formation of a localized elevation on the egg surface, the blastodisc. The activation process begins within 15–20 min. after standing in sea water. The platelets disappear suddenly and completely, all within 30–45 sec. Following this, the perivitelline space forms and gradually enlarges, separating the under-surface of the chorion from the outer surface of the egg proper. Throughout all the experiments recorded, the diameter of the chorion, from the first few minutes after the eggs had been released into sea water, has been found to remain constant. The formation of the perivitelline space is accompanied by a corresponding shrinkage of the egg proper. Meanwhile, the peripheral protoplasmic layer slowly moves towards one pole of the egg, so that, by the end of an hour, there is a distinctly formed ovoid blastodisc on the surface of the egg in preparation for cleavage and subsequent development.

The eggs were always punctured within a few minutes after stripping, at which time the platelets were still present. Upon puncturing, the platelets remained for about half a minute, and then within the next 15 sec. suddenly disappeared completely. Immediately following this, the perivitelline space appeared and gradually enlarged. As a sign of persistence of the normal state of the egg, there was a full development of a blastodisc. The one observable difference from spontaneous activation was in the time of the formation of the blastodisc; after the puncture, it always developed earlier than in spontaneously activated eggs. This difference was explained as due to the fact that puncture always initiated the streaming movements which culminated in the formation of the blastodisc. Since the puncture was always made prior to the beginning of spontaneous activation, the time of the full formation of the blastodisc was by that much earlier. In the cases recorded, this accelerated period averaged 10 min. In general, eggs irreversibly injured, mechanically or otherwise, become opaque throughout. The above findings of activation, together with

the fact that the punctured eggs remained yellow and translucent, were indications that puncturing had no deleterious effect. In one control experiment, an egg was punctured shortly after it was inseminated at the time of stripping. The egg was kept under observation till after five normal cleavages had occurred, while all through this time the puncturing pipette had been kept in place.

In a successfully performed puncture, a small amount of coagulum always formed on the surface of the egg around the shaft of the pipette. This coagulum served as an airtight seal and also to hold the pipette in place. It was this tight seal and the inelasticity of the chorion which made the recording of pressure possible. Of incidental interest was the existence of a relation between the site of puncture and that of the subsequently forming blastodisc. In either NaCl or KCl alone, the blastodisc always formed at the site of puncture. In sea water and in a mixture of NaCl and KCl (19:1), isotonic with sea water, there was no relationship (Kao, 1951).

B. Internal hydrostatic pressure

In Fig. 2 are shown the results of ten experiments on the eggs from different females on different days. Immediately upon puncture there was recorded a negative pressure in the egg averaging about -10 mm. Hg. This negative pressure

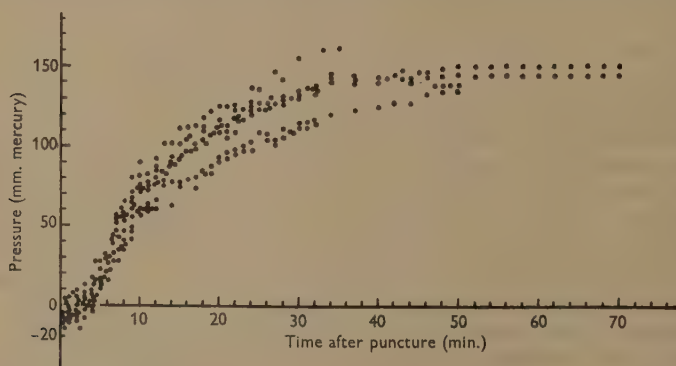


Fig. 2. Internal hydrostatic pressure of puncture-activated eggs in sea water. Each dot represents a measurement on one egg consistently kept in sea water. Beyond 40-50 min., only a few as recorded were kept in sea water. Other eggs used at this time were subjected to other media. The results obtained are recorded in a second paper of this series. (Chambers, Chambers & Kao, 1951).

gradually decreased till about 4 min. later when it was at zero (i.e. atmospheric pressure). At this time the platelets had disappeared for about 3 min., and the perivitelline space had become just visible. During the succeeding 10 min. there was a rapid increase in pressure, averaging about 12 mm. per minute, to that at the end of the first 10 min. the average pressure was about 70 mm. Hg. From 10 min. thereafter, the rise in pressure became progressively slower, until at 50 min. when the pressure reached and remained constant at about 150 mm. Hg. Generally

speaking then, the formation of the internal hydrostatic pressure can be divided into two stages, a rising stage during the first 50 min. after activation, and a long-lasting plateau stage which follows (Kao, Chambers & Chambers, 1951).

Associated with the rise in pressure was the progressive enlargement of the perivitelline space. Dimensions of the various parts of the egg are presented in Table 1. Measurements, by means of an ocular micrometer, were made of the

Table 1. *Dimensions of the various components of the egg at different pressures*

(Two illustrative cases, 201 and 202, are recorded here.)

Egg no.	Time (in min.)	Pressure (mm. Hg)	Volume (mm. ³)			% of whole egg as perivit. space
			Chorionated egg	Egg proper	Perivit. space	
201	0	—	3.2	3.2	0	0
	3	—	3.2	2.53	0.67	20.9
	8	56	3.2	2.19	1.01	31.3
	20	125	3.2	2.16	1.04	32.5
	41	150	3.2	1.75	1.45	45.3
202	0	—	3.6	3.6	0	0
	3	12	3.6	3.0	0.6	15.9
	12	87	3.6	2.9	0.7	19.4
	22	118	3.6	2.35	1.25	34.7
	32	133	3.6	2.19	1.41	38.6
	44	145	3.6	2.19	1.41	38.6

diameter of the whole egg (including the chorion) and that of the egg proper. Volumes of these two parts were then calculated, and the volume of the perivitelline space was obtained by subtraction. Since the volumes of the different parts in different eggs varied appreciably, the volume of the perivitelline space was finally expressed as a percentage of the volume of the entire egg including the chorion. This method of measurement, i.e. calculating volumes from diameters, was used because the various components measured were practically spheres. During the early phase in which the experiments were performed, the blastodisc had not accumulated enough to distort the spherical appearance of the egg proper. From Table 1 it can be seen that the chorion does not alter its dimensions, and that the enlargement of the perivitelline space was due to a decrease in volume of the egg proper.

Correlating the pressure curve (Fig. 2) with Table 1, a question arose as to whether the rise of the internal hydrostatic pressure was due to compression of the egg proper by the expanding perivitelline space. To ascertain the possibility of some perivitelline compression, a method was employed to remove the pressure. Eggs stripped into sea water were allowed to activate spontaneously for 1 hr. After the perivitelline space was fully developed the chorion was snipped* so as to permit a free communication between the perivitelline space and the external medium. When micropipettes were inserted into such eggs, the recorded pressures were never

* We are much indebted to Dr J. P. Trinkaus (Department of Zoology, Yale University) for his generous help in this operation.

above 2–4 mm. Hg. Normally, without snipping the chorion, the pressure at this time would have been in the vicinity of 150 mm. Hg.

Another series of experiments was performed for the purpose of continuous recording of pressure changes. These are represented in Fig. 3, in which one egg was punctured in sea water in the usual manner. All the visible changes, including

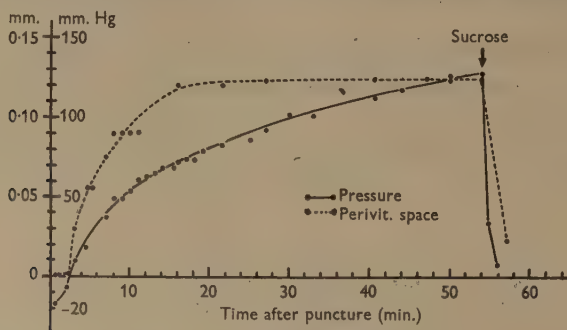


Fig. 3. The progressive changes in pressure and perivitelline space of one egg in sea water and effect of subsequent immersion in sucrose hypertonic (1.9M) to sea water. Sea water was removed and sucrose added when the pressure and perivitelline space have reached a nearly full state of development. The scale on the left side of the ordinate indicates the average width of the perivitelline space as measured by an ocular micrometer. The scale on the right side of the ordinate indicates pressure in mm. of mercury.

the enlargement of the perivitelline space, were the same as those described above. When the pressure and the perivitelline space had reached nearly the full state of development, the external medium was changed from sea water to 1.9 M-sucrose (twice the tonicity of Woods Hole sea water). Immediately the perivitelline space disappeared as the egg proper swelled. Accompanying these morphologic changes was the precipitous fall in pressure which persisted at zero.

IV. DISCUSSION AND CONCLUSION

A. Terminology

Concerning the various coats enveloping the fish egg, the chorion—which is the tough outermost shell covered with fibrous strands—is the only enveloping structure with a generally accepted designation. According to some descriptions, there are altogether two, at most three, enveloping structures, the chorion, the vitelline membrane, and Chambers' protoplasmic surface film constituting the actual surface layer of the egg proper. The two latter have sometimes been identified as one, namely, a vitelline membrane because it encloses the egg proper, formerly known as the vitellus. No accurate location of a vitelline membrane independent of the chorion or of the surface of the egg proper has ever been defined. In our experiments on the *Fundulus* egg, we identified only the chorion and the protoplasmic surface of the egg proper which, when the egg is fertilized or otherwise activated, become separated by a perivitelline space.

B. Discussion of results

The interpretation of the experimental results herein reported are based on the progressive morphologic changes, the progressive pressure change, and the experiments in which the pressure was reduced. The *Fundulus* egg is isotonic with the body fluids of the fish, and when first laid is hypotonic to sea water. Owing to the relative hypertonicity of sea water, some water is lost by the egg during the first few minutes. This would account for the initial negative pressure recorded (Fig. 2). The amount lost, however, is not large enough to induce any appreciable volume change of the egg proper. As the activation process commences with the sudden disappearance of the platelets, the pressure begins to rise. The correlation between the disappearance of the platelets and the development of pressure is further supported by the observation that in those eggs in which the platelets did not disappear, no pressure ever developed. Furthermore, when the platelets disappeared more slowly than normal, the pressure also climbed at a correspondingly slower rate. This condition of slower disappearance of platelets can be produced by immersing the puncture-activated unfertilized eggs from the beginning in isotonic NaCl.

Concerning the formation of the perivitelline space, there must be an accumulation of water between the chorion and the egg proper. In spite of Loeb's work (1912), there is evidence (Bodine, 1928; Armstrong, 1928) that water and electrolytes can traverse the chorion freely. The accumulation of water in the perivitelline space must be attributed to some other component which cannot pass out through the chorion. This substance, which is formed on activation when the platelets disappear, and presumably exudes from the egg proper into the perivitelline space, can be seen as a thick jelly-like material on tearing open the chorion. This jelly-like material imbibes water until saturation at 50 min. after puncture. The morphological changes in the *Fundulus* egg are comparable to those observed in the egg of another teleost, *Oryzias latipes*, by Yamamoto (1939, 1940 1941), who also noticed colloidal material to flow out when the chorion had been opened. The imbibition of water by such a material would explain the observed facts of a progressively enlarging perivitelline space, an accompanying increase of the internal hydrostatic pressure and a corresponding decreasing size of the egg proper. That the recorded pressure in the *Fundulus* egg is due to the perivitelline substance is conclusively shown by the decrease of pressure when the perivitelline space is abolished either mechanically or by immersion in solutions of a non-electrolyte (sucrose). From the maximum pressure in normal environment, this colloidal osmotically active material in the perivitelline space is estimated as equivalent to 0.01 M, which is probably a fairly accurate value because of the great dilution. It is interesting to note that this value compares favourably with the value obtained by Svetlov (1929) for the perivitelline fluid of the trout egg by freezing-point depression, the latter being 0.02° C. The nature of the platelets and their contents has not yet been ascertained. They stain with Sudan III, indicating a lipid nature. Svetlov demonstrated a positive Millon reaction with the perivitelline substance of the trout egg. Possibly the perivitelline

substances of the marine *Fundulus* egg and of the fresh-water trout egg may be closely related or even be identical substances.

The vitelline membrane, whatever it is, of the *Fundulus* egg proper has been claimed to be impermeable to water. It is the purpose of this paper and following papers to show that the methods employed by previous workers showed only an absence of a net exchange of water, and did not indicate non-penetration. Gray (1932), in discussing the impermeability of the trout egg, had already stated that if the vitelline membrane were permeable to water, then water exclusion by the egg must be due either to the development of a hydraulic pressure in the opposite direction or to some active metabolic process. He concluded, however, that neither mechanism was at play, emphasizing especially that no pressure could develop in the egg during life. The reasons for his conclusion were that the chorion was a non-viable component of the egg, and that being freely permeable to water and electrolytes of the external medium, the chorion could not serve as a semipermeable membrane for any osmotic function. The results reported here show clearly that a pressure does develop in the *Fundulus* egg during life, although the pressure is dependent only on the parts external to the living egg proper, viz. the chorion and the colloid in the perivitelline space. Conceivably a similar situation may exist in the trout egg. In any case, results herein reported have shown the development of an opposing hydrostatic force which, owing to its circumferential location around the egg proper, could prevent further ingress or egress of water into or from the egg proper.

Table 1 shows that the shrinkage of the egg proper from the rigid chorion amounts to approximately 40% of the original volume. This can be accounted for only by the loss of something from the egg proper, presumably water, which by moving into the perivitelline space, and kept there by the jelly-like material, contributes to the development of the pressure. Further evidence that the egg proper is permeable to water is provided by the sucrose experiment. The fact that in the sucrose experiment the perivitelline space disappears indicates that not only is the chorion freely permeable to water, but because the diameter of the chorionated egg remains constant, and because the change in volume of the perivitelline space corresponds strictly to the change in volume of the egg proper, it is to be concluded that water transfer to and from the perivitelline space must be through the egg surface out of and into the egg proper.

SUMMARY

By means of a mercury manometer connected to a micropipette, the internal hydrostatic pressure of the unfertilized *Fundulus* egg was measured. Upon activation of the egg by micropuncture when the platelets in the cortex of the egg proper disappeared, there was a progressively increasing internal pressure which finally stabilized at 150 mm. Hg. The pressure was accompanied by the formation of a perivitelline space which contained a clear, colloidal material. The measured internal hydrostatic pressure of the egg was diminished by any means which decreased the

perivitelline space. It was concluded that the perivitelline substance is the chief factor contributing to the pressure. The changes in volume of the egg proper are inversely proportional to the size of the perivitelline space, indicating that the surface of the egg proper is permeable to water.

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